Charles University in Prague, Faculty of Science

Department of Cell Biology

Study programme: Special Chemical and Biological Programmes

Branch of study: Molecular Biology and Biochemistry of Organisms

Zuzana Ezrová

Genes participating in response to *Leishmania major* revealed by targeted mutations

Geny účastnící se odpovědi k *Leishmania major*, zjištěné pomocí cílených mutací

Bachelor's thesis

Supervisor: doc. Marie Lipoldová, CSc.

Institute of Molecular Genetics, ASCR, v. v. i.

Prague, 2014

Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 15. 5. 2014

.....

Zuzana Ezrová

Acknowledgement

I would like to express my gratitude to my supervisor doc. Marie Lipoldová, CSc. for her professional guidance, sincere approach and valuable critiques. I also appreciate the opportunity to work with colleagues from the Laboratory of Molecular and Cellular Immunology and I am thankful for a grant support of Czech Science Foundation GACR 1430-186S. Finally I would like to kindly thank my family and friends for their full support and encouragement.

Content

List of abbreviations

Ahr	Aryl hydrocarbon receptor
AIL	Advanced intercross line
APC	Antigen presenting cell
Bcl-6	B-cell lymphoma 6
CCL19	Chemokine (C-C motif) ligand 19
CCR2	C-C chemokine receptor type 2
CR3	Complement receptor type 3
CD	Cluster of differentiation
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ENU	N-ethyl-N-nitrosourea
Fas (APO1)	Apoptosis antigen 1
GATA-3	Trans-acting T-cell-specific transcription factor GATA-3
GIPL	Glycoinositolphospholipid
gp	Glycoprotein
HIV	Human immunodeficiency virus
HSV-tk	Herpes simplex virus-tyrosin kinase
Ier3	Immediate early response 3
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
Itgam	Integrin alpha M
JAK	Janus kinase
JNK1	c-Jun N-terminal kinase 1
KO	Knock-out
Lgals3	Lectin, galactose binding, soluble 3
LPG	Lysophosphoglycan
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein (CCL2 - Chemokine (C-C motif) ligand 2)
MHC	Major histocompatibility complex
MIF	Migration inhibitory factor
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation marker 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NO	Nitric oxide
PCR	Polymerase chain reaction
PKC	Protein kinase C
QTL	Quantitative trait loci
RA	Receptor antagonist
RBP-J	Recombination signal binding protein for immunoglobulin kappa J
RGEN	RNA-guided engineered nuclease
RIS	Recombinant inbred strain

RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
TALEN	Transcription activator-like nuclease
T-bet	T box transcription factor
TCCR	T-cell cytokine receptor
TCR	T-cell receptor
TGF-β	Transforming growth factor beta
TLR	Toll like receptor
TNF	Tumour necrosis factor
TRAF	TNF receptor associated factors
Treg	Regulatory T-cell
WT	Wild type
ZFN	Zinc finger nuclease

List of figures

List of tables

Abstract

Leishmania major is an intracellular parasite which often successfully multiplies and disseminates in a body of the host thanks to strategies that help it to escape the components of immune system of the host organism. Thus, the parasites evoke an impairment of regulatory pathways that in physiological conditions lead to an expression of genes involved in a response to *L. major* and its efficient elimination. Gene targeted deletion, also called gene knock-out, can result in phenotypic alteration and associated enhanced susceptibility or resistance of the host. Although such detected genes do not have to signify their variability in population and hence they may not be responsible for the worsened outcome of leishmaniasis of some people necessarily, studies in which they are analysed and general knowledge being also a subject of this thesis help us together with techniques of forward genetics to reveal the biochemical pathways during the infection and their elements that influence the outcome of the disease and might be useful for researches of new medicine drugs or gene therapy.

Key words: *Leishmania major*, susceptibility, resistence, targeted mutation, knock-out, immune response, Th cell, cytokines

Abstrakt

Leishmania major je intracelulární parazit, kterému se v mnoha případech daří úspěšně množit a šířit se v těle hostitele. Děje se tak díky strategiím, pomocí nichž *Leishmanie* unikají složkám imunitního systému daného organismu a způsobují narušení regulačních drah, jež za fyziologických podmínek vedou k expresi genů zapojených v odpovědi na tohoto parazita a jeho úspěšné eliminaci. Cílená genová delece, takzvaný genetický knock-out, se může projevit změnou fenotypu a s ním spojenou změnou vnímavosti daného hostitelského organismu. Přestože takto detekované geny nemusí být v populaci polymorfní a nemusí tak být nutně zodpovědné za zhoršený rozvoj leishmaniózy u některých lidí, jejich studie a přehled, jehož zpracování je náplní této práce, mohou společně s metodami přímé genetiky pomoci určit biochemické dráhy a jejich prvky ovlivňující celkový projev onemocnění a mohou tak usnadnit výzkumy pro tvorbu léčiv či genovou terapii.

Klíčová slova: *Leishmania major*, vnímavost, rezistence, cílená mutace, knock-out, imunitní odpověď, Th buňka, cytokiny

1 Introduction

Leishmaniasis is an infectious disease originated from several *Leishmania* species, for instance *L. major, L. donovani and L. braziliensis*, whose uncontrolled replication leads to cutaneous, mucocutaneous or visceral syndromes causing e. g. formation of skin and organ lesions, destruction of mucous membrane and cartilage in nasal cavity and mouth or worse hypertrophy of spleen and liver. It is spread in tropical or dry climate areas mainly in India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil in case of visceral leishmaniasis while cutaneous leishmaniasis appears in Americas, Mediterranean areas and Middle East to Central Asia (Alvar et al., 2012).

Leishmania parasites are intracellular pathogens invading macrophages and other phagocytes to multiply, extend over the body and finish their life cycle. Even though *Leishmania* parasites have some escaping mechanisms, in majority of cases, the immune system promotes sufficient signalling for Th1 response characterized by elevated levels of pro-inflammatory cytokines (IFN-γ, IL-12) and decreased cytokines produced by Th2 cells and other immune compounds. Beside others, the balance between Th1/Th2 cell development and effective parasite elimination is supposed to be influenced by genetic predispositions.

There are two possible genetic approaches nowadays that can be efficiently used together to clarify the presence of products of genes involved in immune response to various pathogens and their function and might promise an effective design of treatment. Apart from gene knock-outs which may confirm possible significant role of a particular gene in regulation of the outcome of disease, methods of forward genetics that can not only determine a range of genes responsible for the susceptibility but also reveal new ones that participate in concrete biochemical pathways are recently applied. It is also a subject of study in the Laboratory of Molecular and Cellular Immunology, ASCR, headed by doc. Marie Lipoldová, CSc. who determine QTLs, regions of chromosomes that control specific manifestations of the disease, by genotyping specifically bred strains e. g. recombinant congenic strains and by comparing their distinct phenotypes (Havelková et al., 2006; Kobets et al., 2012; Sohrabi et al., 2013; Šíma et al., 2011and others). Still, it is necessary to consider all the aspects of vector-parasite-host interactions and their individual features as the laboratory designed experiments cannot fully mimic the natural diversity. Moreover, a problem is that people all over the world are exposed to different environmental and living conditions which also have an impact on their robustness and overall health and not to forget the outcome of the disease resulting from the response of immune system of the host to *Leishmania* parasites is influenced by many genes of minor effects thus it is demanding to recognize an effect of a single gene only.

This thesis is aimed to summarize studies of gene targeted null mutations that influence the response to *L. major* in order to understand defensive pathways of immune system which are important not only in response to *Leishmania* parasites but also other intracellular pathogens.

2 Leishmaniasis

Leishmaniasis is an infectious disease caused by intracellular parasites of *Leishmania* genus. It is transmitted by bites of sand flies that have become infected when ingesting a blood meal from another infected mammal host. The vectors can be located in a damp habitat as well as in subtropical and tropical areas all over the world. Specifically, the illness is spread by *Lutzomya* in the Americas and by *Phlebotomus* in the Old world (Africa, India, south Europe and Middle East) (Herwaldt, 1999).

According to the data collected during 5 or less year periods in years from 2002 to 2010 leishmaniasis threatens over 350 million people living worldwide and there are 98 countries on 5 continents where it appears constantly (Fig. 1). Moreover, a number of 0.9 to 1.6 million new cases and up to 40 thousand deaths may occur annually but it is important to contemplate that it is an estimation only as there is a lack of required information especially in countries where most people are infected (Alvar et al., 2012). Apart from genetic predispositions, leishmaniasis is related to worsened life conditions, malnutrition and lack of professional medical care and depleted immune system which is possibly caused by mentioned issues or also some associated diseases such as HIV (Alvar et al., 1997).

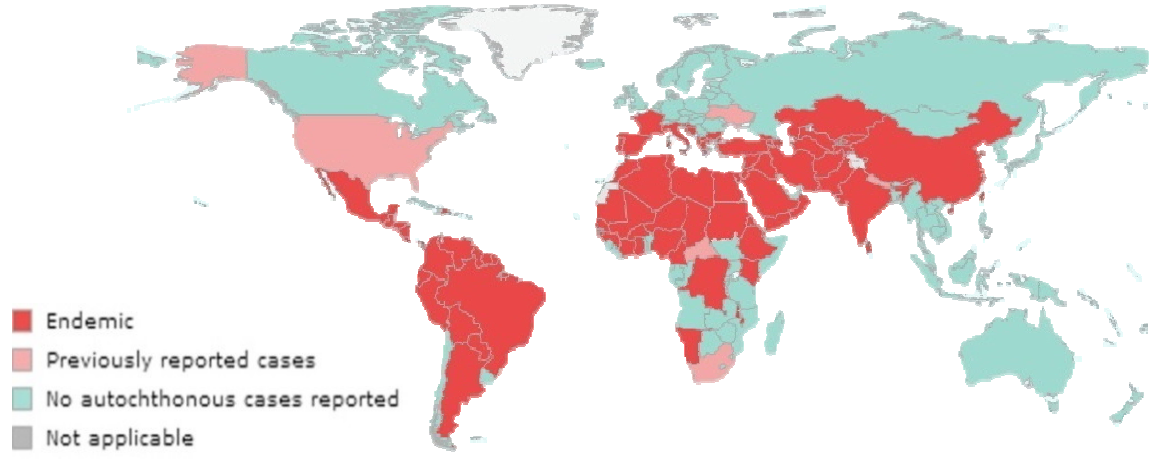


Figure 1: Status of endemicity of cutaneous leishmaniasis, 2013
The figure is taken from http://apps.who.int/neglected_diseases/ntddata/leishmaniasis/leishmaniasis.html

2.1 Disease agents

Concerning significant variability in host's immune response, diversity of vectors and *Leishmania* parasites manifestations of the disease vary from most frequent cutaneous forms, for instance self-healing skin ulcers, to damage of nasal and oral mucosa and cartilage. If the most severe form, visceral leishmaniasis, occurs the parasites migrate through blood and lymph vessels and destroy hematopoietic and filtering organs or nodes. This leads to weakness, fever, weight loss and overall immune system crash possibly including anaemia (Herwaldt, 1999). When visceral leishmaniasis

disappears it might relapse as so-called post-kala-azar dermal leishmaniasis, chronic form incurred by *Leishmania donovani* (Herwaldt, 1999). Thus, we can roughly separate the manifestations into the following groups: cutaneous predominantly due to *L. major, L. tropica and L. aetiopica* in the Old world and *L. braziliensis, L. mexicana and L. amazoniensis* etc. in Americas. Next, mucocutaneous leishmaniasis located mostly just in South America provoked by *L. braziliensis, L. amazoniensis, L. mexicana* and visceral form generated mainly by *L. donovani* in the Old world but also by *L. infantum, or L. tropica* (Murray et al., 2005).

2.2 Life cycle of *Leishmania major*

Leishmania parasites can grow into two morphologically distinct life stages (Fig. 2). First, the non-motile amastigotes are ingested by the sand fly from infected blood of a host. Afterwards, amastigotes differentiate into flagellated procyclic promastigotes in the sand fly's midgut. As promastigotes divide they simply transform themselves into the metacyclic phase and migrate to a pharyngeal valve whence they are injected to another host. Metacyclic promastigotes can actively invade macrophages and granulocytes. Later, they convert into amastigotes that multiply inside the cells and leave after the burst of the cells into blood vessels. Hence, the amastigotes can both attack other cells and escape to another carrier with the blood meal and mature into the infectious promastigotes eventually (Murray et al., 2005; Sacks et al., 2002).

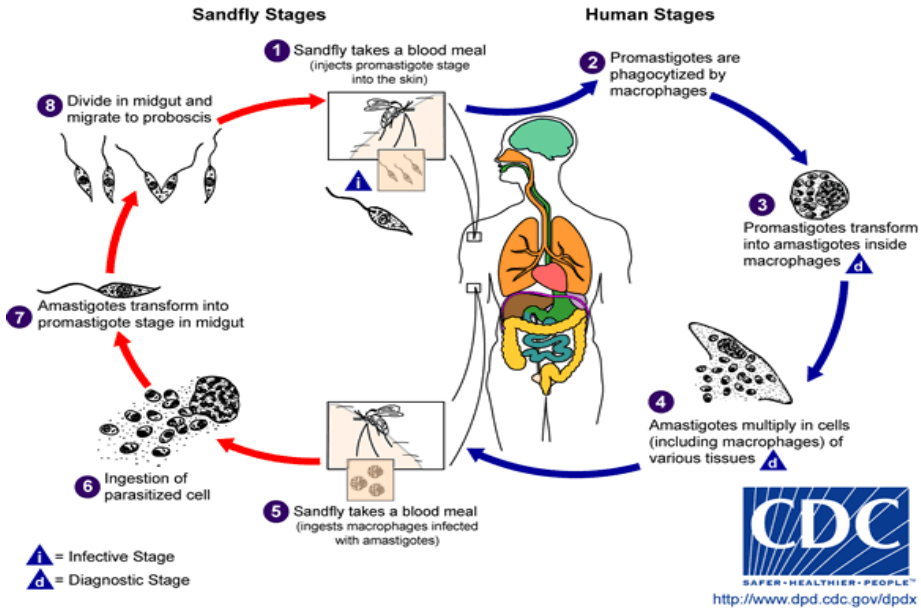


Figure 2: Life cycle of *Leishmania*
The figure is taken from http://www.cdc.gov/parasites/leishmaniasis/biology.html

2.3 Escape strategies

Leishmania parasites are intracellular protozoa, Kinetoplastida, Trypanosomatida. Kinetoplast, a special mitochondria structure of these organisms, contains extra nuclear genetic information for mitochondria proteins as so-called maxi-circles and genes for complex system of proteins as mini-

circles that modify just exactly the DNA of maxi-circles. In other words, in order to obtain functional mRNA transcribed from maxi-circles, it is necessary to correct the primary transcript by RNA editing via proteins coded in the mini-circles (Lukeš et al., 2002).

Aside from the kinetoplast *Leishmania* parasites can be characterised by other extra molecules principally used against host's immune system as escape mechanisms. In the view of fact that *Leishmania* parasites create two life stages, extracellular promastigotes in the sand fly's gut and intracellular amastigotes inside phagocyte cells of mammals, it is crucial for them to remain in such a diverse environment successfully. Thus, they evolved sophisticated and complex system of surface molecules, inhibitors and stimulators against the defence of both the vectors and the hosts in order to not only survive but also reproduce and spread effectively.

Surface molecules

The parasite penetrates into the skin from vector's midgut when being in a stage of metacyclic promastigote. It is not able to actively fight against compounds of immune protection but it can escape efficiently thanks to a thick layer of glycocalyx consisting of numerous glycoproteins, especially lysophosphoglycans (LPGs), glycoinositolphospholipids (GIPLs), glycoproteins 63 or for instance other proteases, whose roles in the process of evading the immune system are still a subject of research (Silvestre et al., 2009).

Expression of LPG differs depending on the particular life stage and its background. Therefore, metacyclic promastigotes capitalize on the thick coat probably due to the protection against transmitter's hydrolases and produce longer and more branched LPGs in comparison to procyclic promastigotes. This is optimal for them to persist, chiefly, the lysis initiated by complement. Amastigotes have the expression of LPG even down-regulated and it is thus believed that its role as a protection against immune system is rather insignificant during this life stage (Pimenta et al., 1991 as cited in Olivier et al., 2005).

Induced phagocytosis

Apart from LPG, there can be found a protease complex gp63 disrupting an extracellular matrix and cleaving an opsonin C3b on the surface of *Leishmania* parasites hence causing an inability of the immune system to assemble complement convertase followed by cell lysis. Therefore, by this transformation the phagocytosis is stimulated via secure receptors, meaning, without an activation of macrophages. This safe path occurs also when LPGs interact with C-reactive protein and its receptor (Olivier et al., 2005).

As the opsonised particle binds to the receptor and is phagocytised, a vesicle, phagosome, sprouts. Following a fusion of phagosome and lysosome, the pH decreases markedly which leads to digestion of the particle. Subsequently, cleaved molecules are degraded by oxygen radicals after an

activation of respiratory burst via protein kinase C or by cytokine-induced nitrite oxide production (Olivier et al., 2005). How *Leishmania* parasites manage to defend themselves against these mechanisms is yet not completely understood. It is possible that an important role is played by surface molecules as the transmembrane residue of LPG connects to another residue of phosphatidyl inositol bisphosphate and thus inhibits activity of PKC (and so NADPH oxidase) (Olivier et al., 1992 as cited in Olivier et al., 2005). They might also resist thanks to the induction of cytokines that cause the iNO synthase suppression and inconvenient stimulation of Th2 immune pathway (Olivier et al., 2005) or thanks to peroxidoxines and superoxide dismutase of *Leishmania* that negatively affect reactive oxide intermediates (Ghosh et al., 2003). There is also a chance that cytosolic perforine, so-called leishporin, creates pores in the membrane of phagocytes resulting in potential outflow of amastigotes into the cytosol (Noronha et al., 2000).

2.4 Efficient immune response to the infection

The immune response to exogenous structures is based on an interaction between elements of innate immunity, NK cells (recognizing MHC class I), phagocytes and complement, and of adaptive immunity using specific molecules that react to every individual component by activation of T and B lymphocytes and creation of antibodies (Fig. 3).

The human immune system literally competes against parasite's interests. As soon as the parasite overcomes mechanical barriers successfully and enters the body of the host, it provokes an inflammatory response and thus the tissue damage. Primarily, ever-present neutrophil granulocytes followed by macrophages, complement and dendritic cells are assembled to the site of inflammation.

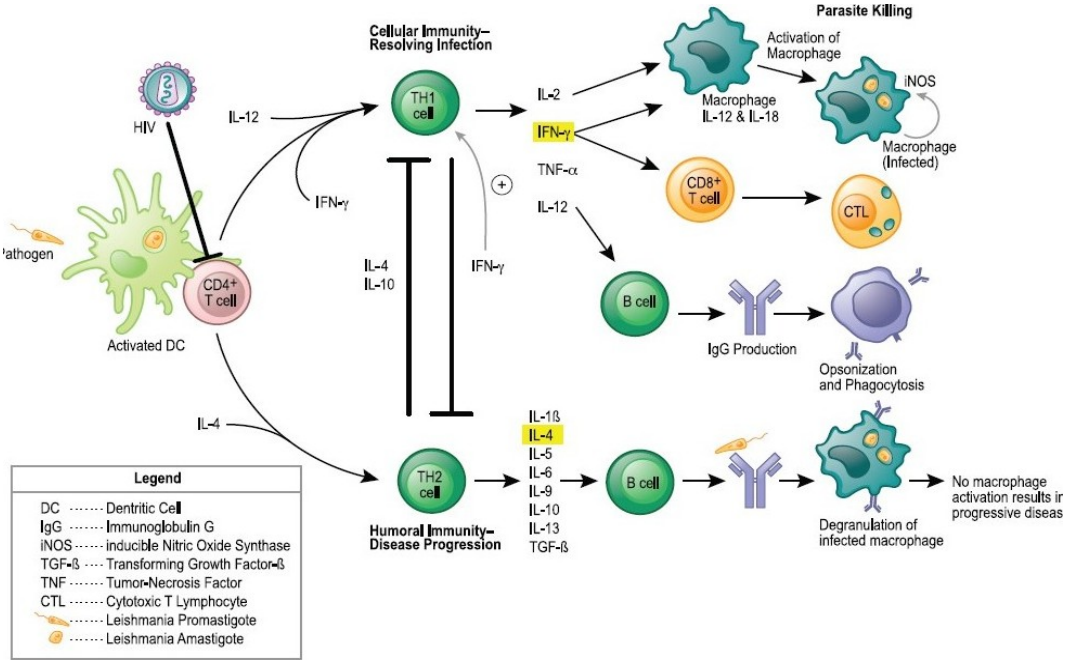


Figure 3: Th response to *L. major* (effect of HIV virus also visible)
The figure is taken from (Ezra, et al., 2010)

Pathogen is therefore effectively defeated by hydrolytic enzymes, respiratory burst or complement-activated lysis plus all these processes are intensified by secretion of supporting cytokines (Mutiso et al., 2013).

Macrophages or dendritic cells can also act as antigen presenting cells (APCs). Such APCs present an antigen to T lymphocytes via MHC glycoproteins especially when the nonspecific immune system fails. Hence, both of the parts of immunity associate and work together.

Naïve precursors of T lymphocytes, pro-thymocytes, develop in thymus into $CD4^+CD8^+$ double-positive T cells with T-cell receptors (TCRs) and CD4/CD8 co-receptors on their surface. After an adhesion of MHC-antigen, the co-receptors are filtered and mature $CD4^+$ or $CD8^+$ T helper (Th) lymphocytes form. Further differentiation of Th lymphocytes into Th1 or Th2 cells is triggered by cytokine signalling and affinity among the antigen and TCR (Miosge et al., 2005). In fact, the balance between Th1 and Th2 response can significantly affect the outcome of the disease. When *Leishmania major* infection expands it was demonstrated that a pathway of IL-4-producing Th2 cells first evolves (Reiner et al., 1994). Nevertheless, it is necessary to shift this pathway in Th1 direction initiated by MHC class II and antigen conjunction. This contact helps the TCR receptors to recognize the co-receptor-MHC II-antigen complex on the surface of APCs leading to IL-12 release. These processes stimulate Th1 cells to produce IFN-γ and other related cytokines resulting in activation of STAT and T-bet transcriptional factors as well as macrophages and in an amplification of these signals heading to self-healing (Sacks et al., 2002). Consequently, it can be assumed that any disruption of production of substances mentioned above contributes to higher susceptibility against leishmaniasis.

2.5 Susceptibility against *Leishmania* parasites

At first the distinct outcome of leishmaniasis was supposed to be a consequence of different ability to generate an adequate inflammatory Th1 response, however, there are studies noticing an explanation of susceptibility only by Th1/Th2 polarization of immune response insufficient. Anderson et al. examined the outcome of the disease caused by *L. major* Sd strain isolated from a patient with non-healing lesions. Surprisingly C57BL/6 mice considered to be resistant also showed non-healing lesions, ulcers and high parasite burdens. Moreover, it was discovered that these mice infected with low dose of 1000 promastigotes had evolved Th1 response as higher level of IFN-γ and corresponding lower levels of IL-4 and IL-13 were detected and the induction of iNO synthase and mobilization of both $CD8^+$ and $CD4^+$ T cells was not altered. As there was no significant difference between Sd and V1 strain of *L. major* identified and a following experiment with mice deficient in IL-10 gene still resulted in inability to fully eliminate the infection, higher susceptibility might have been a result of activity of other cytokines (Anderson et al., 2005). Likewise, a study by Noben-Trauth et al. of BALB/c mice deficient in IL-4 which is believed to be crucial for Th2 response development unexpectedly revealed no significant improvement in a control of the infection and mice infected with

2×10^6 *L. major* LV39 promastigotes failed to resolve the infection (Noben-Trauth et al., 1996). Similar conclusions are indicated in studies of gene KOs registering except for Th1/Th2 dichotomy other components of signalling pathways participating in the disease manifestation.

In any case, $CD4^+$ T lymphocytes which differentiate into IL-4 producing Th2 cells contribute markedly to worsened outcome of the disease as the Th2 development and simultaneous restriction of Th1 cells result in inhibition of APC's function and so the production of IL-12. Moreover, IL-4 and other related cytokines, e. g. IL-6, IL-10, IL-13 and TGF-β (Sacks et al., 2002), stimulate B cells to produce antibodies that are aimed to extracellular pathogens rather than intracellular ones and thus they are useless in the defence. Exactly, high levels of immunoglobulins, IgG, IgM, IgE and IgD (Gosh, et al., 1995 as cited in Mutiso et al., 2013), are detected mainly in mice suffering visceral leishmaniasis when parasites successfully spread into inner organs. This contributes to aggravated capability to resist and interfere against the infection (Mutiso et al., 2013).

A cellular environment surrounding the site of inflammation, meaning which cells are more likely to be instigated to reproduction and differentiation is also involved in increased sensitivity. It includes neutrophils, for example, and other granulocytes that persist in profusion for a long time in the inflammatory site of more susceptible BALB/c when compared to resistant C57BL/6 strain where few neutrophils appear in the site of inflammation temporarily (Beil et al., 1992). In addition, if neutrophils were removed the Th2 direction and manifestation of the disease in BALB/c mice were weakened (Tacchini-Cottier et al., 2000).

Another important role is played by receptors, IL-12R especially, their ligands and cytokines and other effector molecules whose various affinity or even inability of binding affects mechanisms linked in the response to infection naturally (Sacks et al., 2002).

Dysfunction of T regulatory (Treg) cells, the T lymphocytes that provide immune supervision and tolerance to endogenous antigens, is also associated with the course of leishmaniasis. Even though these cells generally influence autoimmune diseases, their competence to produce IL-10 is important not only during leishmaniasis but also other infections. That is why Treg cells might support parasites' long term endurance in the body (Belkaid et al., 2002).

It is obvious that both the resistance and susceptibility are not dependent on selection of inflammatory or humoral response only. It is a sophisticated complex, a network, of cellular and non-cellular components, their mutual relations and stimulations of transcription factors and other molecules. All these substances are coded in genes that can be monogenous as well as polygenous and can exist in linkage or other relations. Therefore it is apparent that polymorphisms existing in the population in these genes may have an impact on the course of leishmaniasis. It is yet a subject of many researches to determine which genes are responsible for the outcome of the disease but as a

mouse-deficient model has been presented and used together with gene mapping a great improvement has been marked.

3 Methods

3.1 Recent genetic approaches

Nowadays, there are two methods used for defining genetic basis of diseases, forward and reverse genetics. Forward genetics seek candidate genes on the ground of unusual phenotype by genotyping inbred strains that could be additionally mutated to raise their mutagenesis rate and variability and by mapping the QTLs. The mapped range of genes that is responsible for specific phenotypic characteristic may be narrowed to one particular gene in this way. However, it is possible and efficient to apply reverse genetics too when we hypothesize the exact gene to be involved. Thus we modify it and analyse the effects of the gene alteration on the resultant phenotype which can reveal us whether the gene of our interest is indeed in the pathways that regulate the outcome of the disease (Ermann et al., 2012).

Forward genetics

A change of the phenotype might be induced in forward genetics by random mutagenesis and hence we can discover plenty of new genes participating in a response to specific pathogen quite quickly in comparison to naturally occurring spontaneous mutations. The most frequent methods of mutagenesis are application of X-rays or some chemical mutagens such as N-ethyl-N-nitrosourea (ENU) or the ability of transposons to transfer and insert a DNA sequence into the genome and cause a mutation by cut and paste or copy and paste mechanisms is also widely used (Weyden et al., 2011).

To map genes included in a response to diseases such as leishmaniasis, special mouse strains differing in size and frequency of parental DNA segments in the genome are cross-bred to make the detection of the genes and specification of their position on the chromosomes easier. For example, recombinant inbred strains (RIS) are therefore generated by crossing two homozygous strains whose genetically identical heterozygous descendants of F1 generation are inbred to make new homozygous strains carrying 50 % of the genes of one parent and 50 % of the other one. Unlike the RIS, recombinant congenic strains are formed by backcrossing the progeny of two inbred strains usually for 20 generations or more with exactly one of the homozygous parent resulting in a creation of new strains containing 12.5 % of the genome of the donor and 87.5 % of the genome of the acceptor. To generate advanced intercross lines (AILs) the strains for the crossing are selected in a way to retain maximum possible number of recombination so the parental DNA segments in the genome are shorten which helps to map the genes more precisely. Another possible option is for instance a use of heterozygous stocks, mouse strains that are created by crossing several inbred parental strains (Demant, 2003).

Reverse genetics and gene targeting

The targeted modification of nuclear genetic information being a tool of reverse genetics was first successful when injecting plasmid DNA of HSV-tk (Herpes simplex virus - thimidine kinase) using glass needle into the cell nucleus where it incorporated into the genome as a result of homologous recombination. Contrary to previous experiments the incorporation of the vector with a specific sequence into the gene of interest and transition of this modified genetic information to the next cell generation has been successful (Capecchi, 2001).

Gene knock-out

One of the most common methods of reverse genetics used for exploring the metabolic pathways is gene knock-out where homologous recombination, use of replacement or insertion vectors, integration of adjusted DNA into the embryo-derived stem cell and additional selection via specific markers in the vector and their inducers are the key steps. Better analysis is ensured by the opportunity to choose the modified cells through the positive-negative selection (Fig. 4). Apart from homologous sections and specific sequence inserted, this filtration is allowed thanks to a sequence for antibiotic resistance (e. g. neomycin) and a virus gene, HSV-tk which is attached to one end of linearized vector. Therefore, we can pick all of the cells with incorporated vector (i. e. positive selection via neomycin) and at the same time we can filter out the cells where the vector integrated non-homologously (negative selection via antiviral HSV-tk). These designed cells can be injected into embryonic stage of blastocyst and transferred to a foster mother. Chimeras generated eventually are backcrossed with both the homozygotes and heterozygotes in order to achieve wild type and heterozygous phenotype as well as homozygous mice that have both of the alleles modified (Capecchi, 2001).

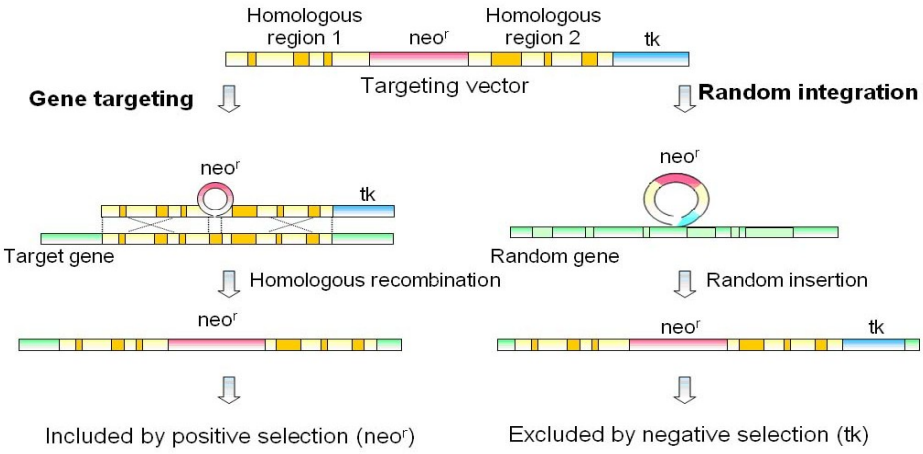


Figure 4: Positive-negative selection. Homologous recombination with the target gene introduces only neo^r; whereas random integration with random gene inserts both neo^r and tk.

The figure is taken from http://proj.ncku.edu.tw/research/commentary/e/20071116/2.html

Today several options of targeted mutations exist. Apart from the gene knock-out other methods such as subtle mutations by hit and run mechanism, when the vector is excised with the targeted gene after its integration or so-called knock-in of the targeted gene, when we can basically exchange any gene for the intended one are in use (Weyden et al., 2002).

Site-specific DNA recombination

Site-specific DNA recombination with a vector that is integrated into the genome of the host and contains extra sequences for regulators of the transcription is also amply implemented, especially a system of Cre site-spesific recombinases (Weyden et al., 2011) that was also applied in two studies of gene deficient mice infected by *L. major* (Auderset et al., 2012, Kinjyo et al., 2006) mentioned in this thesis.

Compared to the knockout the gene of our interest is not completely inactivated but it is usually replaced by its analogue. Specific Cre recombinase or some other recognizes loxP sequences inserted by homologous recombination into the recipient which hence becomes floxed. The gene for Cre recombinase, that generally contains a promoter activated in specific tissue only or in a presence of specific stimulating substance, is brought into the second recipient. When both mice, one of them is floxed and the other has Cre gene incorporated, are crossed the targeted gene is cut out only in the site of effective transcription of Cre recombinase (Weyden et al., 2002).

Although this method is quite exacting it allows a prevention of embryos from extinction caused by potentially lethal null mutation when realizing the classical knock-out. It also decreases the probability of deleted gene's compensation mediated by another gene. In other words, it is possible to generate numerous mutations and examine their impacts that may differ in various tissues. But there is still a potential problem with an occurrence of natural recombination through cryptic loxP-like sequences (Weyden et al., 2002).

RNA interference

A silencing of gene transcription via RNA interference is also worth mentioning. The down-regulatory pathway is activated by DICER enzyme after recognition of RNA (e. g. shRNA) introduced into the genome of the host that pairs with the targeted DNA sequence. DICER cleaves such dsRNA to siRNA which associates with RISC and then together they degrade other mRNAs complementary to such siRNA (Gao et al., 2007).

Programmable nucleases

The latest procedures that may once replace time- and finance- consuming knock-outs and their variations are genome editing via programmable nucleases. Zinc-finger (ZF) and newer transcription activator like (TALE) and RNA-guided engineered (RGE) nucleases differing in

targetable location produce site-specific double strand breaks and then induce the correction by non-homologous end joining mechanism leading to targeted alteration of the genome, point mutations or even chromosomal aberrations such as inversions or translocations. In addition following the creation of double strand breaks, more accurate homologous recombination is possible in case of realization of DNA insertion via vector into the genome of the host (Kim and Kim, 2014).

It is remarkably helpful to understand how both the pathogen and host defend their interests and how exactly their individual proteins and other molecules interact while searching for the vaccine. Techniques of forward genetics together with gene targeting yet achieves probably the most effective results when identifying candidate genes involving symptoms of numerous diseases including leishmaniasis and role of human immune system.

3.2 In vivo infection with *Leishmania major*

Methods of analysis of gene deficient mice infected with *L. major* vary among different research groups. They may choose distinct mouse strains as well as diverse parasite's species which may have an impact on the results they obtain as resistance to *L. major* of some strains might be dependent on early IL-12 production and of others rather not (Demant, 1996).

Mouse strains

Despite the mouse model offers great advantages to study molecular and genetic processes thanks to its relative similarities with human genome, short life cycle and easy manipulation (Weyden et al., 2002), the research is often aimed to the mouse models only thereby mouse-human differences and distinct biochemical pathways of individuals are substantially omitted. It is thus very convenient to examine other mouse strains with diverse genetic background apart from the most widely used susceptible BALB/c and resistant C57BL/6 because it would in fact take account of naturally occurring variability in human population (Ermann et al., 2012). Examples might be susceptible MAI (Babay et al., 2004) or P/J, SWR/J a CcS-16 (Lipoldová et al., 2006) strains and resistant PWK (Babay et al., 2004) or B10.D2, CBA, C3H, STS, 129/Sv, CcS-5 (Lipoldová et al., 2006). Following mouse strains were used in studies described here of gene KOs examined during *L. major* infection.

C57BL

C57BL and its substrain C57BL/6 also called black 6 (B6) for its black-coloured hair is probably the most frequent inbred strain used in numerous experiments. C57BL was firstly inbred by the founder of Jacksons Laboratory C. C. Little (The Jackson Laboratory, 2014). It is widely used thanks to its availability, strain stability and easy breeding. The strain is also known for its resistance against tumour growth, convenient environment to express modified genes and increased inflammatory response. When infected with *L. major* it shows significant resistance and ability to heal. In comparison to other strains such as BALB/c or DBA/2J it lives longer. C57BL helps not only in

immunology and parasitic diseases but also in diabetes, obesity, neurobiology and cardiovascular fields of study. Individual strains may differ in order to laboratory to be generated in (e. g. Jacksons and Harlan laboratories) (Festing, 1998).

129

Most substrains of 129 are light or white-coloured and thus they are often crossed with dark C57BL/6 mice when doing gene targeting since it is easy to find out whether the incorporation of mutation was successful by observing the hair colour of such designed transgenic mice. It is applied in immunology and cancer research as several lines of embryo-derived stem cells are easily accessible, however, 129 mice have poor breeding performance and the substrains genetically vary (Festing, 1998).

C3H

C3H mice used in various researches of cancer and infectious diseases included are also resistant to *L. major* and have a high rate of spontaneous mutations that also occurred in C3H/HeJ substrain at the lipopolysaccharide locus making it more resistant to endotoxin while other strains are endotoxin sensitive. Majority of subtrains have a good breeding performance and exhibit very low aggression only (Festing, 1998).

BALB/c

BALB/c are albino mice highly sensitive to *Leishmania* parasites (and other pathogens e. g. some species of *Salmonella, Helicobacter or Trypanosoma*) that are unable to control the infection and hence the infection might lead to fatal visceral form. Despite some substrains (for instance BALB/cJ) are very aggressive, they are used for examining abnormalities of immune response considering their susceptibility against several pathogens and for creating recombinant congenic strains when mapping genetic variability (Festing, 1998).

Parasites

It has been proven in a study of Dr. Nancy Noben-Trauth who had discovered apart from probable minor role of IL-4 in response to *L. major* (see above) that IL-4R$\alpha^{-/-}$ deficient BALB/c mice infected with *L. major* LV39 strain remain susceptible whereas mice infected with IR173 strain were highly resistant and in some cases they could control the infection even better than C57BL/6 mice (Nobe-Trauth, 2000). The outcome of the disease hence depends on several aspects including mouse and parasite species thus it is necessary to take them all into consideration when preparing an experiment.

The number of inoculated parasites has been showed to be important as well. Using real-time PCR, the Laboratory of Parasitic Diseases and Immunology led by Nicola Kimblin attempted to determine the number of parasites that can be transmitted into the host by single vector. If an intake of

blood meal and transmission that could be detected via fluorescent markers in parasites occurred, the number of transferred promastigotes ranged between ten to hundreds and thousands. Approximately 75 % of infected mice contained less than 600 parasites possibly as a result of interruption during the intake. The remaining 25% of mice showed more than 1000 promastigotes which might be the cause of blood meal's passive back-flow in the vector rather than a formation of a plug made from gel secreted by parasites (Kimblin et al., 2008).

When analysing the impact of parasite's dose injected into mice in laboratory conditions, high doses evoked lesion creation immediately while low doses surprisingly displayed more parasites persisting in the body of the host and thus created new reservoir. The research is rather aimed to more frequent cutaneous leishmanisis and as it is performed in artificial environment the higher doses used in many experiments seem to be adequate (Kimblin et al., 2008).

Promastigotes were inoculated intradermally or subcutaneously into different parts of mouse body, most commonly into the ears and hind footpads in all studies discussed in this thesis and the dose of parasites injected into mice varied from 10^2 to 10^7 but most often it was 10^6 *Leishmania* parasites.

4 Targeted mutations enhancing the susceptibility against *L. major*

An overview of the gene KOs described here with details of mouse and parasite strains and the outcomes of leishmaniasis can be found in the attachment as well as a map of signalling pathways influenced by these genes and illustration of genes' approximate positions on chromosomes.

4.1 Genes involved in Jak/Stat signalling pathway

APCs produce specific cytokines influencing the differentiation of $CD4^+$ T helper cells into Th1 or Th2 lymphocytes. Such lymphocytes direct the immune response against *Leishmania* parasites by secreting distinct signal molecules. IFN-γ together with IL-12 provide the Th1 response direction and its maintenance as they activate the macrophages and inhibit Th2 cells simultaneously leading to an efficient elimination of intracellular parasites of *Leishmania* or other genus as well (Wang et al., 1994). One of the first works that used gene targeted null mutation to clarify Th1/Th2 dichotomy of immune response to leishmaniasis concerns immediately the role of IFN-γ in defence mechanisms.

Ifng

The research group of Wang et al. from University of California analysed progression of the disease in mice on C57BL/6 resistant background with *Ifng* gene knocked out and infected with 10^6 promastigotes inoculated into the hind footpads (Wang et al., 1994). Infected mice developed large lesions gradually, hypertrophy of lymph nodes and spleen and even sudden death of some mice occurred 5 to 7 weeks post infection most probably due to fully expanded Th2 response since higher levels of IL-4, IL-5, IL-13 cytokines and IgG1 and IgE antibodies have been detected (Wang et al.,

1994). In physiological conditions IFN-γ triggers a phosphorylation cascade by activating Janus kinases JAK1 and JAK2 leading to a translocation of STATs into nucleus where T-bet transcription factor that inhibits IL-4 and IL-5 production and at the same time strengthens its own transcription is expressed (Thomson Reuters, 2013). On the basis of this signalling pathway the results have showed markedly enhanced susceptibility to *L. major* as expected and crucial role of IFN-γ in Th differentiation as well as in later control of infection and disease resolution.

Socs1

Suppressors of cytokine signalling (SOCSs) are expressed when stimulated with IFN-γ whose production they supress afterwards and thereby negatively regulate JAK/STAT pathway (Bullen et al., 2003). Thus, when SOCS1 is absent an overproduction of IFN-γ contributing to overall resistance of the host might be presumed. Increased level of IFN-γ and concurrently decreased levels of IL-4 and IL-10 in mice on resistant background deficient in one copy of *Socs1* infected with 10^4 promastigotes inoculated intradermally into the pinna of the ear were detected indeed (Bullen et al., 2003). In spite of the presence of cytokines indicating Th1 immune response these mice surprisingly displayed persisting lesions and delayed healing phenotype in comparison to wild type (WT) mice. Furthermore, if mice with both copies of *Socs1* deleted were infected they died very soon, in most of the cases in 2 to 4 weeks post infection (Bullen et al., 2003). *Socs1* KO promotes an activation of IL-12 induced STAT4 and IL-4 induced STAT6 that stimulate both Th1 (STAT4) and Th2 (STAT6) cells (Fujimoto, et al., 2002) plus in mice lacking SOCS1 there was no significant alteration of Th2 cytokine levels observed (Bullen et al., 2003). Together these data suggest a role of SOCS1 in regulation and maintenance of the balance of cytokine signalling mediated by both Th1 and Th2 cells probably during parasites' persistence rather than at a time of T cell differentiation (Bullen et al., 2003).

Socs3

To analyse immune response to *Leishmania* parasites in *Socs3*$^{-/-}$ deficient mice on resistant background infected with 10^7 promastigotes inoculated subcutaneously in the right footpad Cre-LoxP system of targeted mutations was used (Kinjyo et al., 2006). In comparison to WT mice, higher parasite burdens and increased levels of IL-10 and TGF-β leading to delayed healing were noticed in mice missing SOCS3, however, footpad swelling, IFN-γ and IL-4 production were comparable (Kinjyo et al., 2006). This implies dominant generation of Th3 cells that secrete large amounts of exactly TGF-β and weaken both Th1 and Th2 lymphocytes. Therefore, suppressive activity of SOCS3 that inhibits both IL-12 mediated STAT4 and IL-10 mediated STAT3 signalling via suppression of JAK and primarily Th3 differentiation is necessary to control the infection (Kinjyo et al., 2006).

Stat1

STAT1, a signal transducer and activator of transcription 1, is activated mainly by IFN-γ but also by other JAK1 mediated cytokines (for instance IL-27 or IL-6) that induce JAK/STAT

cooperation. This interference results in expression of IL-12, TNF and iNOS too which all take significant part in an elimination of *Leishmania* parasites (Späth et al., 2009).

Rosas et al. aimed their research to investigate IFN-γ mediated STAT1 signalling pathway and its task in a response to *L. major* hence *Stat1*$^{-/-}$ deficient mice on resistant C57BL/6 background were infected with 2×10^6 promastigotes injected in the hind footpad. The infection manifested lesions even larger than those observed in highly susceptible BALB/c mice (Rosas et al., 2003). Moreover, in a similar experiment parasites could survive easily inside a phagolysosome of *Stat1* deficient mouse macrophages as a phagosomal acidification was disrupted most likely due to a dysfunction of chloride channels (Späth et al., 2009). There were greater amounts of IL-10 and less but detectable IFN-γ and IL-12 monitored in lymph node cells thereby a loss of control over dissemination of parasites occurred following disarrayed production of NO in macrophages (Rosas et al., 2003). As IL-4 production was not affected even though in the absence of STAT1 increased levels might have been awaited, these results suggest an activity of JAK/STAT independent signalling and its ability to produce some IFN-γ that can supress Th2 response firstly but later is not sufficient enough and fails to resolve the infection totally. Thus, the outcome of the disease and control over the infection are caused not only by IFN-γ dependent STAT1 signalling leading to an activation of T-bet transcription factor but also by other mechanisms that are at least partially capable of compensation of this pathway (Rosas et al., 2003).

Stat4

The previous studies of Kinjoyo and Rosas research groups mentioned above showed that the progress of leishmaniasis is not dependent on Th1/Th2 differentiation only. Another laboratory that had examined the role of IL-12 activated STAT4 that just as STAT1 induces IFN-γ production also came to this conclusion (Stamm et al., 1999). Following an inoculation of 2×10^6 promastigotes in the hind footpads of *Stat4*$^{-/-}$ deficient mice generated on resistant background, larger progressive non-healing lesions and parasite burdens developed when compared to WT. The presence of IL-12 and small amounts of both IFN-γ and IL-4 indicate an existence of STAT4 independent IL-12 pathway which stimulates T lymphocytes into Th1 response despite the KO of this signal regulator. Yet, the alternative signalling was not sufficient as in the previous case of STAT1 deficiency and thus the lack of STAT4 resulted in enhanced susceptibility to *L. major* (Stamm et al., 1999).

Il27r

IL27R, also called T-cell cytokine receptor (TCCR) or WSX-1, is a receptor of cytokine family class I. Yoshida et al. explored the outcome of leishmaniasis in *Il27r*$^{-/-}$ deficient mice backcrossed on resistant background and infected with 5×10^6 promastigotes injected subcutaneously in the right hind footpad (Yoshida et al., 2001). The course of infection was greatly worsened in comparison to WT, nevertheless, BALB/c still exhibited more severe signs of the disease. Low level of IFN-γ had been noted 2 weeks post infection but it raised and corresponded to the production of

IFN-γ in WT mice after 5 to 6 weeks (Yoshida et al., 2001). It is likely that IL27R is essential during initial stages of infection although it is dispensable later on. The presence of IgG1 and IgE antibodies signifies substantial activity of Th2 cells (Yoshida et al., 2001) but it was rather an attenuation of STAT1 signalling and associated disruption of Th1 and T-bet stimulation which is regularly supported by IL27R during the polarization of immune response (Takeda et al., 2003).

Il6

Resistant *Il6*$^{-/-}$ deficient mice were infected with 10^4 promastigotes of *L. major* or alternatively of *L. major* with CpG DNA inserted because Mendez, Wu et al. had discovered that the infection with this genetically modified strain exhibited more moderate outcome of the disease in their preceding study and hence they have decided to include it in the analysis of *Il6* KO. Surprisingly both of the strains of *L. major* caused a formation of large non-healing lesions and necrosis of the tissue in the site of infection (Wu et al., 2009). Contrary to WT, the level of IL-10 was elevated possibly because of intensified proliferation of IL-10 secreting Treg cells. KO mice infected with *L. major* alone showed the most aggravated course of the infection from all tested mouse models used in this experiment which might be explained by relative success of application of modified parasites that did not affect the production of IFN-γ and IL-12 that much (Wu et al., 2009). Considering the positive influence of IL-6 on JAK/STAT signalling pathway as well as on NF-κB factor (Thomson Reuters, 2013) we may predict its role in a defence to intracellular parasites in initiating STAT dependent pathways exactly heading to an activation of T-bet or NF-κB mediated pro-inflammatory cytokines.

Bcl6

BCL6, B-cell lymphoma 6, regulates the differentiation of T lymphocytes by shifting Th1/Th2 response and also by an alteration of STAT signalling (Dent et al., 1999). The function of *Bcl6* in immune system during leishmaniasis was inspected by generating its null mutation in resistant mice infected with 10^6 promastigotes subcutaneously in the right hind footpad. Numerous skin ulcers, necrosis of tissue and comparable parasite loads as well as typical Th2 cytokines, IL-4, IL-5 and IL-13, appeared in KO mice just as in BALB/c but the production of IFN-γ was not that affected (Dent et al., 1999). These results clearly demonstrate a development of Th2 cells and enhanced susceptibility that was in accordance to the influence of BCL6 to GATA-3 which stimulates an expression of mentioned cytokines in physiological conditions. The role of IL-4 seemed to be rather minor as the outcome of the disease in *Bcl6*$^{-/-}$*Il4*$^{-/-}$ double KO mice displayed nearly the same levels of IL-13 and IL-5 as in *Bcl6*$^{-/-}$ KO but was exacerbated when compared to WT or *Il4*$^{-/-}$ KO. Dent et al. had performed other experiments and proved a dependence of enhanced susceptibility on STAT6 which is predominantly induced by IL-13 and whose absence in *Bcl6*$^{-/-}$ deficient mice converted vulnerable ones into resistant (Dent et al., 1999).

4.2 Genes involved in NF-κB and MAPK signalling pathways

Nfκb

Various stimuli can initiate a cascade of reactions leading to a deactivation of IκB followed by a stimulation of NF-κB which helps to express other factors whether they stimulate cells to proliferate, urge them to an angiogenesis or a tissue invasion or pass on a survival signal (Artis et al., 2003). Such complexity of this signalling and all the compounds involved suggests a significant position in immunity. The task of NF-κB during leishmaniasis was investigated by Artis et al. from University of Pennsylvania, Department of Pathobiology on *Nfkb1*$^{-/-}$ deficient mice backcrossed on resistant C57BL/6 background inoculated with 5×10^6 promastigotes injected in the hind footpad. The infection culminated in chronic non-healing lesions apparently due to reduced proliferation of antigen specific $CD4^+$ T cells producing IFN-γ (Artis et al., 2003). There were no noteworthy changes in production of neither IL-12 nor NO detected in comparison to WT but when *Nfkb2* KO was realized the production of NO was impaired. Altogether, the susceptibility of mice lacking NF-κB2 might be a cause of decreased IL-12 secretion while in mice lacking NF-κB1 disrupted signalling to the proliferation via molecules such as IL-1, IL-6 or TNF necessary for a correct immune response of $CD4^+$ T cells is probably responsible (Artis et al., 2003).

Ccr2

One of such impulses that activate NF-κB is chemokine receptor CCR2. KO of *Ccr2* in mice backcrossed on the resistant background and infected with 2×10^6 promastigotes inoculated intradermally into both ears seems to play an important role in maturation and migration of dendritic cells and resultant production of IL-12 and thus Th1 differentiation (Jimenez et al., 2010). Actually, the level of IL-12 was markedly reduced as well as the expression of CCL19 chemokine and MHC class II which is located on the surface of DCs and that are both responsible for proper behaviour and migration of DCs. Therefore, the enhanced susceptibility to leishmaniasis is also related to the function of CCR2 which moves NF-κB to translocate into the nucleus and so influences the expression of other effector molecules (Jimenez et al., 2010).

Il1ra

IL-1 receptor antagonist (IL-1RA) blocks an access of IL-1 to its receptor and restrains negative effects of IL-1 overexpression as its signalling via TRAF6 supresses NF-κB thus the outcome of leishmaniasis should be worsened in IL1RA deficient mice theoretically (Voronov et al., 2010). Indeed, elevated levels of IL-4, IL-6, IL-10 and IL-17 cytokines and connected aggravation of infection were observed in comparison to *Il1*KO and even BALB/c (Voronov et al., 2010). Similar conclusions were deduced when *Il1ra* KO was performed on BALB/c mice infected with 10^3 promastigotes inoculated intradermally in ear skin as they also exhibited enhanced susceptibility associated with low levels of IFN-γ, IL-12, increased levels of Th2 cytokines and with more

neutrophils and DCs accumulated in the site of infection causing partial damage of tissue (Kautz-Neu et al., 2011).

Notch

Notch1 and Notch2 receptors regulate both GATA-3 and NF-κB factors through recombining binding protein suppressor (RBP-J) thereby they influence differentiation and proliferation of T lymphocytes and production of specific cytokines. In case of only one of the *Notch* being knocked out, mice were resistant to *L. major* even though *Notch1* is more likely to be the dominant one as its expression prevailed over *Notch2* in control mice. Double KO in mice on C57BL/6 genetic background was examined after an exposure to 3×10^6 promastigotes injected in the footpad (Auderset et al., 2012). As a consequence of the deficiency of both Notch receptors contributing to low IFN-γ and high IL-5 and IL-13 content T cells were not able to produce enough IFN-γ and fully develop Th1 response. There is also an indication for a direct effect of Notch on IL-4 promoter because there was no modulation found in comparison to IL-5 and IL-13 levels although all of these cytokines are under the control of GATA-3 (Auderset et al., 2012).

Lgals3

Galectin 3 plays a role in numerous cellular functions including apoptosis, innate immunity, cell adhesion and T-cell regulation and it also acts similarly as TLRs. The absence of Lgals3 in BALB/c mice infected with *L. major* provoked in strengthened proliferation of IL-10 secreting Treg cells (Fermino et al., 2013). A crucial role of IL-10 alone in a response to *L. major* was introduced in a study of its KO in BALB/c mice leading to enhanced resistance (Kane et al., 2001). Alike in already mentioned experiment of Wu et al. with *Il6*$^{-/-}$ C57BL/6 mice, increased secretion of IL-10 in mice lacking galectin 3 resulted in enhanced sensitivity and in this case influencing expression of Notch receptors. In addition Treg cells were present at the site of infection in profusion conducing to a creation of lesions. The migration of Treg cell to the site of infection might be related to raised activity of DCs as Lgals3 attenuates it normally (Fermino et al., 2013).

Cd40l

Glycoprotein CD40 ligand expressed on a surface of $CD4^+$ T lymphocytes takes part not only in humoral immunity when stimulating isotype switching of antibodies but also in cellular immunity where its importance is based on an elimination of infected cells via interaction with receptors of death and an initiation of NF-κB and MAPKs pathways through TRAF factor and subsequent activation of macrophages (Thomson Reuters, 2013). Hence, the KO of *Cd40l* in resistant mice infected with $2 - 5 \times 10^5$ promastigotes in the hind footpad obviously came to defect in IFN-γ, IL-2 and IL-12 production and elevated IL-4 levels. The exacerbated course of infection is therefore caused most probably by failure of Th1 response generation (Campbell et al., 1996).

Myd88

Research group of Muraille et al. seeking for potential factors involved in the outcome of leishmaniasis engaged to Myeloid differentiation marker, MyD88. Resistant mice deficient in this gene and infected with 5×10^6 promastigotes inoculated subcutaneously in the left hind footpad evoked typical Th2 response with phenotype similar to BALB/c (Muraille et al., 2003). MyD88 interferes with TLRs which recognise wide range of pathogens on the ground of common patterns such as LPS and lipoproteins. It is possible that the enhanced susceptibility in MyD88 deficient mice is connected to an impairment of TLRs signalling as the bond between these patterns and TLRs triggers MAPKs and NF-κB pathways through MyD88 heading to the production of cytokines that launch a respiratory burst or Fas-mediated apoptosis of infected cells (Muraille et al., 2003).

Tlr

The hypothesis of TLRs contributing to the outcome of the disease was tested by Fakher et al. from Institute of Pasteur in Paris who analysed functions of TLR2, TLR4 and TLR9 by targeted mutations in mice backcrossed on resistant C57BL/6 background infected with 3×10^6 promastigotes (Fakher et al., 2009). The course of infection with *L. major* worsened in TLR9 deficient mice only. An absence of this gene evinced a suppression of IL-12 and IFN-γ expression and linked inability of efficient response to *Leishmania* parasites (Fakher et al., 2009).

Mif

MIF, migration inhibitory factor, is a cytokine which reduces anti-inflammatory effects and helps to produce TNF and NO. Potentially important role of MIF in defence system of the host against intracellular parasites was the subject of the work by Satoskar et al. implementing null mutation of *Mif* in resistant mice (Satoskar et al., 2001). KO mice infected with 2×10^6 promastigotes inoculated in the hind footpad manifested greater parasite burdens and large non-healing lesions in later stages of infection. Furthermore, the production of cytokines specific for an invasion of intracellular parasite was not modified suggesting that MIF has no impact on the activation and differentiation of T cells. Nevertheless, high IL-6 content was observed (Satoskar et al., 2001). IL-6 can induce the expression of IFN-γ as explained above (Wu et al., 2009) but it can inhibit the expression too when reacting with SOCS3 suppressor. Following the suppression NO production by macrophages may have not been realized which could be the reason of ineffective parasite elimination (Satoskar et al., 2001).

Ras

Individual isoforms of Ras factor are activated by binding to TCR receptors but they have no significant effects on maturation and differentiation of T cells as an experiment of $Ras^{-/-}$ deficient mice on resistant background has showed (Iborra et al., 2011). A low dose of 300 promastigotes inoculated intradermaly into ears of these mice did not alter the production of IL-12 and NO in comparison to WT and the level of IL-4 increased up to several weeks post infection. However, IFN-γ was greatly

reduced due to the disruption of Ras signalling which is involved in proliferation and apoptosis of $CD4^+$ T cells through MAPKs. This experiment as well as many others confirmed the critical role of IFN-γ as one of the most important cytokines in the defence of the host (Iborra et al., 2011).

Jnk1

On the other hand, *Jnk1*$^{-/-}$ mice generated on resistant background infected with 2×10^6 promastigotes inoculated in the right hind footpad were able to develop Th1 immune response as no change of pro-inflammatory cytokines was noted. Yet it was not sufficient enough to repress the activity of Th2 cells that secreted significantly higher amounts of IL-5, IL-13 and Th2 dependent IgG1 antibodies. The inhibition of Th2 cytokines via JNK1 appeared to be necessary in control of leishmaniasis in physiological conditions although the phenotype was not as severe as in BALB/c (Constant et al., 2000).

MhcII

MHC class II is a glycoprotein supporting an interaction between antigen presenting cell and T-cell receptor which binds such complex (antigen-MHC-APC) and starts a cascade of reactions evoking an adequate immune response (Chakkalath et al., 1995). Surprisingly, mice with *MhcII* knocked out backcrossed to C57BL/6 background and infected with 10^5 promastigotes were able to handle the infection at the beginning even better than BALB/c but contrary to WT the level of IFN-γ was decreased. This course is most likely on account of $CD8^+$ T cells and their ability to produce small amounts of IFN-γ which were sufficient in early stages, nonetheless, MHC II deficient mice succumb the infection eventually (Chakkalath et al., 1995).

4.3 Genes involved in apoptotic pathways

IER3, Fas and Fas ligand and TNF are the products of genes that regulate proliferation and cell differentiation and one of their main tasks in immune system is to present a signal of death to infected cells and regulate MAPKs pathway (Akilov et al., 2009; Conceicao-Silva et al., 1998; Wilhelm et al., 2001). Infection of resistant mice lacking the single gene each time did not affect Th differentiation but led to loss of control over the replication of parasites as NO production was disrupted and accumulation of infected macrophages which resulted in a formation of lesions and tissue necrosis at the site of infection (Akilov et al., 2009; Conceicao-Silva et al., 1998; Wilhelm et al., 2001). Moreover at the beginning of infection of *Ier3* deficient mice elevated level of IL-17 was detected which corresponds to its function in recruiting neutrophils to the site of inflammation (Akilov et al., 2009).

5 Targeted mutations enhancing the resistance against *L. major*

Il13

The analysis of effects of *Bcl6* KO already mentioned led not only to clarification of its function during inflammatory response but also to revelation of STAT6 whose lack enhanced the

resistance. STAT6 is induced by IL-4 and IL-13 and it has been also discovered that IL-13 alone is able to modulate the activity of Th cells in contrary to IL-4 of which the deficiency in *Bcl6* KO mice had rather no additive effects on the course of infection (Dent et al., 1999). Thus, Matthews et al. decided to investigate whether the KO of *Il13* has such impact on BALB/c mice infected with *L. major*. Indeed, when IL-13 was absent Th1 cells were not restricted in production of pro-inflammatory cytokines and the mice showed enhanced resistance. Additionally when double $Il4^{-/-}$ $Il13^{-/-}$ KO mice were infected they could control leishmaniasis with ease (Matthews et al., 2000).

Il1

An importance of IL-1 regulation has already been proven in the study of IL-1RA (Kautz-Neu et al., 2011, Voronov et al., 2010). Possible effects of IL-1 which signals an expression of adhesion molecules on endothelial cells and leukocytes albeit its role in immune system is much more complicated were examined in $Il1^{-/-}$ deficient Babl/c mice infected with 10^7 promastigotes subcutaneously at the base of the tail (Voronov et al., 2010). As expected, moderated manifestations of leishmaniasis occurred most possibly thanks to enabled TRAF6-NF-κB signalling leading to an expression of T-bet factor and directing the production of Th1 cytokines, IL-12, IFN-γ and IL-18 that were present in IL-1 deficient mice (Voronov et al., 2010).

Il17

A function of another cytokine, IL-17, was studied on $Il17^{-/-}$ deficient BALB/c mice. Despite Th2 immune response evolved in these mice which was indicated by the levels of IFN-γ, IL-4 and IL-10 cytokines similar to BALB/c WT, the outcome of the disease was much less severe (Kostka et al., 2009). This might be a result of IL-17 ability to recruit neutrophils and DCs to the site of inflammation where the cells accumulate and hence they can disrupt the tissue later on (Akilov et al., 2009, Kostka et al., 2009).

Ccl2

Mice generated on resistant background lacking *Ccl2* chemokine gene (also called *Mcp1*) displayed strengthen defiance during the infection of *L. major* in comparison to WT as no MCP-1 stimulation of the expression of Th2 cytokines, IL-4, IL-5 and IL-10, was therefore achievable (Gu et al., 2000). BALB/c mice with *Mcp1* KO also moderated the symptoms. They had less footpad swelling and were able to stabilize the lesions to some extent so the phenotype they exhibited was intermediate between susceptible and resistant strains (Gu et al., 2000).

Itgam

Apart from principal cytokines it is necessary not to forget the interaction between the parasite and surface molecules on phagocytic cells. For instance it is a chemokine receptor ITGAM (CR3) which binds lysophosphoglycan of *Leishmania* parasites thereby the promastigotes can penetrate into

the cells. In order to this mechanism of invasion when *Itgam* is knocked out the course of infection gets somewhat better but more importantly, part of this receptor, a chain CD11b, transpired to be the one which interacts with various patterns of exogenous pathogens in the view of fact that its deficiency rapidly enhanced the resistance in BALB/c mice as the parasite could not have entered the cells (Carter et al., 2009).

Ahr

Aryl hydrocarbon receptor interferes with STAT1 and NF-κB whose activity is therefore attenuated (Kimura, et al., 2009). In spite of its KO in resistant mice during *L. major* infection that modulated the production of cytokines, as low IL-12 and high IL-10 content was observed at first probably due to increased level of TNF-α, the immune system was capable of developing Th1 response afterwards sufficient enough to resolve the infection (Elizondo et al., 2011).

6 Conclusion

First studies of leishmaniasis attributed enhanced susceptibility to incorrect decision of the immune system of the host to drive Th2 response aimed to extracellular pathogens which was evoked by phagocytosis induced by parasites. Many of the experiments of gene targeted mutations analysed in this thesis led to worsened outcomes or even succumbing to the disease in comparison with WT due to reduced levels of IFN-γ and/or IL-12 or on the contrary because of an overproduction of Th2 cytokines especially IL-4, IL-5, IL-13 and IL-10. Thus, Th1/Th2 polarization is crucial in the response to *L. major* indeed. But it is demonstrated here that there are other processes independent on Th signalling which contribute to manifestations of leishmaniasis. For example it is a defect of chloride channels of phagocytes leading to an increase of phalysosomal pH in STAT1 deficient mice (Späth et al., 2009), an overproduction of IL-1 in mice knocked out in its receptor antagonist (Voronov et al., 2010) or a presence of STAT4 independent signalling (Stamm et al., 1999). These mechanisms are yet not all involved. For instance a work of Kinjyo et al. showed a significant role of SOCS3 whose deficiency resulted in differentiation of T lymphocytes into TGF-β producing Th3 cells that supress both Th1 and Th2 cell activity (Kinjyo et al., 2006). Next, enhanced sensitivity when *Il6* gene was knocked out was a cause of intensified proliferation of IL-10 secreting Treg cells rather than Th2 cells even though Th2 response was in fact evoked (Wu et al., 2009). In addition the research of Satoskar et al. demonstrated an important function of IL-6 inhibiting MIF whose absence did not have any impact on neither Th1 nor Th2 cytokines but still displayed enhanced susceptibility most likely as a consequence of macrophages dysfunction caused by association of IL-6 and SOCS3 (Satoskar et al., 2001). It is also necessary not to forget cell receptors such as ITGAM through which the parasites can enter cells easily and apoptosis signalling genes as their deficiency induces impaired NO production and hence the elimination of parasites as well.

The reality is therefore more complicated than it was supposed initially. The response of the host to *L. major* is a complex network of molecules whose misbalance or single absence may lead to a chain of irreversible reactions causing a successful dissemination of parasites. Knock-outs enhancing both the susceptibility and resistance help us to learn about sophisticated strategies of *Leishmania* parasites as well as genes involved in response to this parasite. As the course of leishmaniasis is directed by a greater number of genes which may be often revealed by QTL mapping, there are many new studies coming out annually that investigate the effects of the candidate genes by their targeted mutations or other methods too. Only this year, analysis by Masoud Akbari et al. of *Irf4* deficiency or by Sachiko Sato et al. explaining the interaction of galectin-3 and surface molecules of *Leishmania* parasites which is also mentioned in this thesis has been published. Resulting from a cooperation of methods of forward and reverse genetics these works can hence be efficiently applied for gene therapy or design of new vaccines to fight not only with *Leishmania* parasites but also other intracellular pathogens and can help us to understand the metabolic pathways and gene expression of their intermediates in response to *L. major* in our immune system.

7 References

Akilov, O. E., Ustyugova, I. V., Zhi, L., et al. Enhanced Susceptibility to Leishmania Infection in Resistant Mice in the Absence of Immediate Early Response Gene X-1. *The Journal of Immunology.* 2009, 189, pp. 7994-8003.

Alcais, A., Abel, L., David, C., et al. Evidence for a Major Gene Controlling Susceptibility to Tegumentary Leishmaniasis in a Recently Exposed Bolivian Population. *The American Journal of Human Genetics.* 1997, 61, pp. 968-979.

Alvar, J., Canavate, C., Gutierrez-Solar, B., et al. 1997. Leishmania and human immunodeficiency virus coinfection: the first 10 years. *Clinical Microbiology Reviews.* 1997, 10, pp. 298-319.

Alvar, J., Velez, I. D., Bern, C., et al. Leishmaniasis worldwide and global estimates of its incidence. *PLOS one.* 2012, 7, e35671.

Anderson, C. F., Mendez, S., Sacks, D. L. Nonhealing infection despite Th1 polarization produced by a strain of *Leishmania major* in C57BL/6 mice. *The Journal of Immunology*. 2005, 174, pp. 2934-2941.

Artis, D., Speirs, K., Joyce, K., et al. 2003. NF-kB1 Is Required for Optimal CD4+ Th1 Cell Development and Resistance to Leishmania major. *The Journal of Immunology.* 2003, 170, pp. 1995-2003.

Auderset, F., Schuster, S., Coutaz, M., et al. Redundant Notch1 and Notch2 Signaling Is Necessary for IFNg Secretion by T Helper 1 Cells During Infection with Leishmania major. *PLOS Pathogens.* 2012, 8, pp. e1002560.

Babay, B. E., Louzir, H., Kebaïer, C., et al. Inbred strains derived from feral mice reveal new pathogenic mechanisms of experimental leishmaniasis due to Leishmania major. *Infection and immunity*. 2004, 72, pp. 4603-4611.

Barbi, J., Snider, H. M., Bhardwaj, N. et al. Signal transducer and activator of transcription 1 in T cells plays an indispensable role in immunity to Leishmania major by mediating Th1 cell homing to the site of infection. *The FASEB Journal.* 2009, 23, pp. 3990-3999.

Beil, W. J., Meinardus-Hager, G., Neugebauer, D. Ch., et al. Differences in the onset of the inflammatory response to cutaneous leishmaniasis in resistant and susceptible mice. *The Journal of Leukocyte Biology.* 1992, 52, pp. 135-142.

Belkaid, Y., Piccirillo, C. A., Mendez, S., et al. CD4+CD25+ regulatory T cells control Leishmania major persistance and immunity. *Nature.* 2002, 420, pp. 502-507.

Bogdan, Christian. Mechanisms and consequences of persistence of intracellular pathogens: leishmaniasis as an example. *Cellular Microbiology.* 2008, 10, pp. 1221-1234.

Bucheton, B., Abel, L., Kheir, M. M., et al. Genetic control of visceral leishmaniasis in a Sudanese population: candidate gene testing indicates a linkage to the NRAMP1 region. *Genes and Immunity.* 2003, 4, pp. 104-109.

Bullen, D. V. R., Baldwin, T. M., Curtis, J. M., et al. Persistence of lesions in suppressor of cytokine signaling-1-deficient mice infected with Leishmania major. *The Journal of Immunology.* 2003, 170, pp. 4267-4272.

Campbell, K. A., Ovendale, P. J., Kennedy, M. K., et al. CD40 ligand is required for protective cell-mediated immunity to Leishmania major. *Immunity.* 1996, 4, pp. 283-289.

Capecchi, Mario R. Generating mice with targeted mutations. *Nature Medicine.* 2001, 7, pp. 1086-1090.

Carter, C. R., Whitcomb, J. P., Campbell, J. A., et al. Complement receptor 3 deficiency influences lesion progression during Leishmania major infection in BALB/c mice. *Infection and Immunity.* 2009, 77, pp. 5668-5675.

Cohen-Tannoudji, M., Babinet, C. Beyond 'knock-out' mice: new perspectives for the programmed modification of mammalian genome. *Molecular Human Reproduction.* 1998, 4, pp. 929-938.

Conceicao-Silva, F., Hahne, M., Schröter, M., et al. The resolution of lesions induced by Leishmania major in mice requires a functional Fas (APO-1, CD 95) pathway of cytotoxicity. *European Journal of Immunology.* 1998, 28, pp. 237-245.

Constant, S. L., Dong, C., Yang, D. D., et al. JNK1 is required for T cell-mediated immunity against Leishmania major infection. *The Journal of Immunology.* 2000, 165, pp. 2671-2676.

Demant, P., Lipoldová, M., Svobodová, M., et al. Resistance to Leishmania major in mice. *Science*. 1996, 274, 1392-1393.

Demant, P. Cancer susceptibility in the mouse: genetics, biology and implications for human cancer. *Nature Reviews Genetics*. 2003, 4, pp. 721-734.

Dent, A. L., Doherty, M. T., Paul, W. E., et al. BCL-6-deficient mice reveal an IL-4-independent, STAT6-dependent pathway that controls susceptibility to infection by Leishmania major. *The Journal of Immunology.* 1999, 163, pp. 2098-2103.

Elizondo, G., Rodríguez-Sosa, M., Estrada-Muniz, E., et al. Deletion of the Aryl Hydrocarbon Receptor Enhances the Inflammatory Response to Leishmania major Infection. *International Journal of Biological Sciences.* 2011, 7, pp. 1220-1229.

Ermann, J., Glimcher L. H. Agter GWAS, mice to rescue? Current *Oppinion in Immunology*. 2012, 24, pp. 564-570.

Ezra, N., Ochoa, M. T., Craft, N. Human immunodeficiency virus and leishmaniasis. *Journal of Global Infectious Diseases.* 2010, 2, pp. 248-257.

Fakher, F. H. A., Rachinel, N., Klimczak, M., et al. TLR9-Dependent Activation of Dendritic Cells by DNA from Leishmania major Favors Th1 Cell Development and the Resolution of Lesions. *The Journal of Immunology.* 2009, 182, pp. 1386-1396.

Fermino, M. L., Dias, F. C., Lopes, C. D., et al. Galectin-3 negatively regulates the frequency and function of CD4+CD25+Foxp3+ regulatory T cells and influences the course of Leishmania major infection. *European Journal of Immunology.* 2013, 43, pp. 1806-1817.

Festing, Michael FW. Inbred Strains of Mice: 129. *Mouse Genome Database.* [Online] 1998. http://www.informatics.jax.org/external/festing/mouse/docs/129.shtml.

Festing, Michael FW. Inbred Strains of Mice: BALB. *Mouse Genome Database.* [Online] 1998. http://www.informatics.jax.org/external/festing/mouse/docs/BALB.shtml.

Festing, Michael FW. Inbred Strains of Mice: C3H. *Mouse Genome Database.* [Online] 1998. http://www.informatics.jax.org/external/festing/mouse/docs/C3H.shtml

Festing, Michael FW. Inbred Strains of Mice: C57BL. *Mouse Genome Database.* [Online] 1998. http://www.informatics.jax.org/external/festing/mouse/docs/C57BL.shtml.

Fujimoto, M., Tsutsui, H., Yumikura-Futatsugi, S., et al. A regulatory role for suppressor of cytokine signaling-1 in Th polarization in vivo. *International Immunology.* 2002, 14, pp. 1343-1350.

Gao X. and Zhang P. Transgenic RNA Interference in Mice. *Physiology.* 2007, 22, pp. 166.

Ghosh, S., Goswami, S., Adhya, S. Role of superoxide dismutase in survival of Leishmania within the macrophage. *Biochemical Journal.* 2003, 369, pp. 447-452.

Gu, L., Tseng, S., Horner, R. M., et al. Control of TH2 polarization by the chemokine monocyte chemoattractant protein-1. *Nature.* 2000, 404, pp. 407-411.

Havelková H., Badalová J., Svobodová M. et al. Genetics of susceptibility to leishmaniasis in mice: four novel loci and functional heterogeneity of gene effects. *Genes and Immunity.* 2006, 7, pp. 220-33

Herwaldt, B. L. Leishmaniasis. *Lancet.* 1999, 354, pp. 1191-1199.

Chakkalath, H. R., Theodos, C. M., Markowitz, J. S., et al. Class II major histocompatibility complex-deficient mice initially control an infection with Leishmania major but succumb to the disease. *The Journal of Infectious Diseases.* 1995, 171, pp. 1302-1308.

Iborra, S., Soto, M., Stark-Aroeira, L., et al. H-ras and N-ras are dispensable for T-cell development and activation but critical for protective Th1 immunity. *Blood.* 2011, 117, pp. 5102-5111.

Jimenez, F., Quinones, M. P., Martinez, H. G., et al. CCR2 Plays a Critical Role in Dendritic Cell Maturation: Possible Role of CCL2 and NF-κB. *The Journal of Immunology.* 2010, 184, pp. 5571-5581.

Kane, M. M., Mosser, D. M. The Role of IL-10 in Promoting Disease Progression in Leishmaniasis. *The Journal of Immunology.* 2001, 166, pp. 1141-1147.

Kautz-Neu, K., Kostka-Lopez, S., Dinges, S., et al. A Role for Leukocyte-Derived IL-1RA in DC Homeostasis Revealed by Increased Susceptibility of IL-1RA-Deficient Mice to Cutaneous Leishmaniasis. *The Journal of Investigative Dermatology.* 2011, 131, pp. 1650-1659.

Kim, H., Kim, J. S. A guide to genome engineering with programmable nucleases. *Nature Reviews Genetics*. 2014, 15, pp. 321-334.

Kimblin, N., Peters, N., Debrabant, A., et al. Quantification of the infectious dose of Leishmania major transmitted to the skin by single sand flies. *Proceedings of the National Academy of Sciences of the United States of America.* 2008, 105, pp. 10125-10130.

Kimura, A., Naka, T., Nakahama, T., et al. Aryl hydrocarbon receptor in combination with Stat1 regulates LPS-induced inflammatory responses. *Journal of Experimantal Medicine.* 2009, 206, pp. 2027-2035.

Kinjyo, I., Inoue, H., Hamano, S., et al. Loss of SOCS3 in T helper cells resulted in reduced immune responses and hyperproduction of interleukin 10 and transforming growth factor–β1. *The Journal of Experimental Medicine.* 2006, 203, pp. 1021-1031.

Kobets, T., Grekov, I., Lipoldova, M. Leishmaniasis: prevention, parasite detection and treatment. *Current medicinal chemistry.* 2012, 19, 1443-1474.

Kostka-Lopez, S., Dinges, S., Griewank, K., et al. IL-17 Promotes Progression of Cutaneous Leishmaniasis in susceptible mice. *The Journal of Immunology.* 2009, 182, pp. 3039-3046.

Lindsay, M. A. Target discovery. *Nature Reviews.* 2003, 2, pp. 831-838.

Lipoldová, M., Demant, P. Genetic susceptibility to infectious disease: lessons from mouse models of leishmaniasis. *Nature Reviews Genetics*. 2006,7, pp. 294-305.

Lukeš, J., Guilbride, L. D., Votýpka, J., et al. Kinetoplast DNA Network: Evolution of an Improbable Structure. *Eukaryotic Cell.* 2002, 1, pp. 495-502.

Matthews, D. J., Emson, C. L., McKenzie, G. J., et al. IL-13 is a susceptibility factor for Leishmania major infection. *The Journal of Immunology.* 2000, 164, pp. 1458-1462.

Miosge, L. A., Goodnow, C. C. Genes, pathways and checkpoints in lymphocyte development and homeostasis. *Immunology and Cell Biology.* 2005, 83, pp. 318-335.

Muraille, E., Trez, C. D., Brait, M., et al. Genetically resistant mice lacking MyD88-adapter protein display a high susceptibility to Leishmania major infection associated with a polarized Th2 response. *The Journal of Immunology.* 2003, 170, pp. 4237-4241.

Murray, H. W., Berman, J. D., Davies, C. R., et al. Advances in leishmaniasis. *Lancet.* 2005, 366, pp. 1561-1577.

Mutiso, J. M., Macharia, J. C., Gicheru, M. M., et al. Immunology of leishmaniasis. *Scientica Parasitologica.* 2013, 14, pp. 51-61.

Noben-Trauth, N. Susceptibility to Leishmania major infection in the absence of IL-4. *Immunology letters.* 2000, 75, pp. 41-44.

Noben-Trauth, N., Kropf, P., Müller, I. Susceptibility to Leishmania major Infection in Interleukin-4-deficient Mice. *Science*, 1996, 271, pp. 987-990.

Noronha, F. S., Cruz, J. S., Beirao, P. S., et al. Macrophage damage by Leishmania amazonensis cytolysin: evidence of pore formation on cell membrane. *Infection and Immunity.* 2000, 68, pp. 4578-4584.

Olivier, M., Gregory, D. J., Forget, G. Subversion Mechanisms by Which Leishmania Parasites Can Escape the Host Immune Response: a Signaling Point of View. *Clinical Microbiology Reviews.* 2005, 18, pp. 293-305.

Reiner, S. L., Zheng, S., Wang, Z.., et al. Leishmania Promastigotes Evade Interleukin 12 (IL-12) Induction by Macrophages and Stimulate a Broad Range of Cytoklnes from CD4+ T Cells during Initiation of Infection. *The Journal of Experimantal Medicine.* 1994, 179, pp. 447-456.

Rosas, L. E., Keiser, T., Pyles, R., et al. Development of protective immunity against cutaneous leishmaniasis is dependent on STAT1-mediated IFN signaling pathway. *European Journal of Immunology.* 2003, 33, pp. 1799-1805.

Sacks, D., Noben-Trauth, N. The Immunology of Susceptibility and Resistance to Leishmania Major in Mice. *Neture Reviews.* 2002, 2, pp. 845-858.

Sarantseva, S. V., Schwarzmann, A. L. Modern Genetic Approaches to Searching for Targets for Medicinal Preparations. *Russian Journal of Genetics.* 2009, 45, pp. 761-770.

Satoskar, A. R., Bozza, M., Sosa, M. R.., et al. Migration-Inhibitory Factor Gene-Deficient Mice Are Susceptible to Cutaneous Leishmania major Infection. *Infection and Immunity.* 2001, 69, pp. 906-911.

Sharma, U., Singh, S. Immunobiology of leishmaniasis. *Indian Journal of Experimental Biology.* 2009, 47, pp. 412-423.

Schwarz, T., Remer, K. A., Nahrendorf, W., et al. T Cell-Derived IL-10 Determines Leishmaniasis Disease Outcome and Is Suppressed by a Dendritic Cell Based Vaccine. *PLOS Pathogens.* 2013, 9, p. e1003476.

Silvestre, R., Santarém, N., Tavares, J., et al. Recognition of Leishmania Parasites by Innate Immunity. *Immunology, Endocrine & Metabolic Agents in Medicinal Chemistry.* 2009, 9, pp. 106-127.

Síma M., Havelková H., Quan L., et al. Genetic control of resistance to Trypanosoma brucei brucei infection in mice. *PLOS Neglected Tropical Diseases.* 2011, 5, pp. e1173.

Sohrabi Y., Havelková H., Kobets T., et al. Mapping the Genes for Susceptibility and Response to Leishmania tropica in Mouse. *PLOS Neglected Tropical Diseases*. 2013, 7, pp. e2282.

Späth, G. F., Schlesinger, P., Schreiber, R., et al. A Novel Role for Stat1 in Phagosome Acidification and Natural Host Resistance to Intracellular Infection by Leishmania major. *PLOS Pathogens.* 2009, 5, p. e10000381.

Stamm, L. M., Satoskar, A. A., Ghosh, S. K., et al. STAT-4 mediated IL-12 signaling pathway is critical for the development of protective immunity in culateous leishmaniasis. *European Journal of Immunology.* 1999, 29, pp. 2524-2529.

Tacchini-Cottier, F., Zweifel, C., Belkaid, Y., et al. An Immunomodulatory Function for Neutrophils During the Induction of a CD4+ Th2 Response in BALB/c Mice Infected with Leishmania major. *The Journal of Immunology.* 2000, 165, pp. 2628-2636.

Takeda, A., Hamano, S., Yamanaka, A., et al. Cutting Edge: Role of IL-27/WSX-1 Signaling for Induction of T-Bet Through Activation of STAT1 During Initial Th1 Commitment. *The Journal of Immunology.* 2003, 170, pp. 4886-4890.

The Jackson Laboratory. JAX® Mice database. *The Jackson Laboratory.* [Online] 2014. http://jaxmice.jax.org/strain/000664.html.

Thomson Reuters. Apoptosis and Survival: Anti-apoptotic TNFS/NF-KB/BCL-2 Pathway. Pathway Maps. [Online] *Thomson Reuters*, 2013. http://pathwaymaps.com/maps/720/.

Thomson Reuters. Immune response: IFN gamma signaling pathway. Pathway Maps. [Online] *Thomson Reuters*, 2013. http://pathwaymaps.com/maps/432/#432_11929133.

Thomson Reuters. Immune Response: IL-6 Signaling Pathway. Pathway Maps. [Online] *Thomson Reuters*, 2013. http://pathwaymaps.com/maps/479/.

Voronov, E., Dotan, S., Gayvoronsky, L., et al. IL-1-induced inflammation promotes development of leishmaniasis in susceptible BALB/c mice. *International Immunology.* 2010, 22, pp. 245-257.

Wang, Z., Reiner, S. L., Zheng, S., et al. CD4+ Effector Cells Default to the Th2 Pathway in Interferon gamma-deficient Mice Infected with Leishmania major. *The Journal of Experimental Medicine.* 1994, 179, pp. 1367-1371.

van der Weyden, L., Adams, D. J., Bradley, A. Tools for targeted manipulation of the mouse genome. *Physiological genomics*. 2002, 11, pp. 133-164.

van der Weyden, L., White, J. K., Adams, D. J., Logan, D. W. The mouse genetics toolkit: revealing function and mechanism. *Genome Biology.* 2011, 12, pp. 224.

Wilhelm, P., Ritter, U., Labbow, S., et al. Rapidly fatal leishmaniasis in resistant C57BL/6 mice lacking TNF. *The Journal of Immunology.* 2001, 166, pp. 4012-4019.

Wu, W., Weigand, L., Mendez, S. The IL-6-deficient mouse exhibits impaired lymphocytic responses to a vaccine combining live Leishmania major and CpG oligodeoxynucleotides. *Canadian Journal of Microbiology.* 2009, 55, pp. 705-713.

Yoshida, H., Hamano, S., Senaldi, G., et al. WSX-1 Is Required for the Initiation of Th1 Responses and Resistance to L. major Infection. *Immunity.* 2001, 15, pp. 569-578.

Secondary sources

Gosh, et al., 1995 as cited in Mutiso, J. M., Macharia, J. C., Gicheru, M. M., et al. Immunology of leishmaniasis. *Scientica Parasitologica.* 2013, 14, pp. 51-61.

Olivier, M. et al., 1992 as cited in Olivier, M., Gregory, D. J., Forget, G. Subversion Mechanisms by Which Leishmania Parasites Can Escape the Host Immune Response: a Signaling Point of View. *Clinical Microbiology Reviews.* 2005, 18, pp. 293-305.

Pimenta, P. F., 1991 as cited in Olivier, M., Gregory, D. J., Forget, G. Subversion Mechanisms by Which Leishmania Parasites Can Escape the Host Immune Response: a Signaling Point of View. *Clinical Microbiology Reviews.* 2005, 18, pp. 293-305.

8 Attachments

Table 1: An overview of performed gene targeted mutations in order of their position on chromosomes with description of mouse and parasites strains and with the outcome of the disease compared to the WT mice

Targeted gene, position	Mouse strain	Parasite	Outcome in comparison with WT unless otherwise stated	References
Stat4 **Chr. 1**, 26,67 cM, 52,008 Mb Signal transducer and activator of transcription 4	C57BL/6×129/Sv	*L. major,* LV39 2×10^6 promastigotes inoculated in the hind footpad	Similar IL-4, IL-12, IgG1 and IgE as WT ↓ IFN-γ, IgG2a Possible Stat4 independent IL-12 signalling pathway but not sufficient enough to develop Th1 response Progressive non-healing lesions that contained significantly higher numbers of parasites Extensive subcutaneous tissue destruction	(Stamm, et al., 1999)
Stat1 **Chr. 1**, 26, 81 cM, 52,119 Mb Signal transducer and activator of transcription 1	129/Sv	*L. major* strain LV39 clone V (Rho/SU/59/P) 10^6 promastigotes inoculated into the footpad	↑ phago-lysosomal pH Defect in Cl channels Progressive non-healing lesions	(Späth, et al., 2009)
	C57BL/6	*L. major*, LV 39 2×10^6 promastigotes into the footpad	↑ IL-10, levels of IL-4, TGF-β not altered ↓ IFN-γ, IL-12, NO Possible IFN-γ independent Stat1 pathway Progressive non-healing lesions Extensive subcutaneous tissue destruction	(Rosas, et al., 2003)
Il1rn **Chr. 2**, 16,36 cM, 24,336 Mb IL-1 receptor antagonist	BALB/c	*L. major* (MHOM/IL/80/Friedlin) 1×10^3 metacyclic promastigotes inoculated intradermally in ear skin of mice (Katz-Neu)	↑IL-6, a IL-17, DCs, neutrophils Normal IL-4, IL-10 ↓ IFN-γ, IL-12 Progressive non-healing lesions 58-fold higher (to WT) parasite number in lesions 8 wk post infection and also increased parasite number in spleens	(Kautz-Neu, et al., 2011)
	BALB/c	*L. major* (MHOM/IL/80/Friedlin) 10^7 promastigotes inoculated subcutaneously at the base of the tail (Voronov)	↑ IL-4, IL-10, IL-6, a IL-17, DCs, neutrophils ↓ IFN-γ, IL-12 Development of nodules Extensive subcutaneous tissue destruction Significant increase in parasite load in both liver and spleen	(Voronov, et al., 2010)

Notch1, Notch2 **Chr. 2** (N1), 18,91 cM, 26,457 Mb **Chr. 3** (N2), 42,24 cM, 98,013 Mb	C57BL/6 Cre-LoxP system used	*L. major* LV39 (MRHO/Sv/59/P strain) 3×10^6 promastigotes were injected in the footpad	↑ IL-5, IL-13 ↓ IFN-gamma Possible direct effect on IL-4 promoter Progressive non-healing lesions Parasite dissemination to the lymph nodes and spleen	(Auderset, et al., 2012)
***Ras, Hras, Nras* isoforms** **Chr. 3** (*Nras*), 45,25 cM, 103,058 Mb **Chr. 7** (*Hras*), 86,48 cM, 141,189 Mb Harvey rat sarcoma virus oncogene, Neuroblastoma ras oncogene	KO mice backcrossed to C57BL/6 background for ≥ 6 generations	*L. major* clone V1 300 metacyclic promastigotes inoculated into the ear intradermaly	Impaired induction of TF T-bet → ↓ IFN-γ Normal IL-4, later increased levels IgG1 (specific for Th2) detected Failure to develop Th1 response Progressive non-healing lesions Increased parasite number in the site of infection and lymph nodes 12 wk post infection	(Iborra, et al., 2011)
Nfkb **Chr. 3**, 62,82 cM, 135,584 Mb Nuclear factor of kappa light polypeptide gene enhancer in B cells	C57BL/6 × 129 background backcrossed to C57BL/6 ≥ 8 generations	*L. major* (MHOM/IL/80/Friedlin) 5×10^6 promastigotes inoculated in the hind left footpad	↓IFN-gamma Without an effect on Th polarization Defect in CD4+ T cells differentiation Large progressive non-healing lesions Extensive subcutaneous tissue destruction Number of parasites in lesions was not significantly greater → possible ability to control parasite replication to some extent	(Artis, et al., 2003)
Il6 **Chr. 5**, 15,70 cM, 30, 010 Mb Interleukin-6	C57BL/6	*L. major* clone VI, *L. major*/CpG (MHOM/IL/80/Friedlin) 1×10^4 promastigotes inoculated intradermally in the ear	↑ IL-10, Treg cells (in both Lm and Lm/CpG infected mice) ↑ IFN-γ, IL-12 (Lm/CpG) ↓ CD4+ T cells, IL-12, IFN-γ (Lm) Progressive non-healing lesions and tissue necrosis Elevated parasite numbers in lesions	(Wu, et al., 2009)
***Il27ra* (*WSX-1*)** **Chr. 8**, 40,26cM, 84,030 Mb Class I cytokine receptor	129/J × C57BL/6 backcrossed to C57BL/6 for ≥ 9 generations	*L. major* (MHOM/SU/73-5-ASKH) 5×10^6 promastigotes inoculated subcutaneously n the right hind footpad	↑ IL-4, IL-13, IgG1, IgE ↓ IFN-γ, but later no impairment in its production Normal IgG2a Progressive non-healing lesions Increased footpad swelling and severe ulceration	(Yoshida, et al., 2001)
Ccr2 **Chr. 9**, 75,05 cM, 124,101 Mb C-C chemokine receptor	KO mice backcrossed to C57BL/6J background for ≥10 generations (or C57BL/6 × 129 → similar results)	*L. major* clone VI (MHOM/IL/80/Friedlin) 2×10^6 or 1×10^7 promastigotes inoculated intradermaly into both ears	↓ IL-12, CCL-19, MHC II Failure to develop Th1 response Impaired recruitment of DCs	(Jimenez, et al., 2010)

Tlr9 **Chr. 9**, 57,49 cM, 106,222 Mb Toll-like receptor-9	KO mice backcrossed to C57BL/6 for ≥ 10 generations	*L. major* LV39 3×10^6 promastigotes inoculated in the footpad	↓ IFN-gamma, IL-12 Inhibition of Th1 response Reduced maturation of DCs Large non-healing lesions present even 7 wk post infection Higher number of parasites in lesions and lymph nodes 4 wk post infection	(Fakher, et al., 2009)
Myd88 **Chr. 9**, 71,33 cM, 119,335 Mb Myeloid differentiation primary response gene 88	KO mice backcrossed to C57BL/6 for 8 generations	*L. major* (MHOM/IR/–/173 strain) 5×10^6 promastigotes inoculated subcutaneously in the left hind footpad	Typical Th2 response ↑ IL-4, IL-10 ↓ IFN-g, IL-12 Progressive non-healing lesions Lesion size similar to observed in BALB/c	(Muraille, et al., 2003)
Mif **Chr. 10**, 38,59 cM, 75,859 Mb Macrophage migration-inhibitory factor	C57BL/6 × 129/Sv	*L. major* LV39 2×10^6 promastigotes inoculated in the hind footpad	↑ IL-6 ↓ NO, O2- No impairment in IFN-g, IL-4, IL-10, IL-12, TGF-β production - no significant impact on Th polarization Macrophage dysfunction Large progressive lesions development Greater parasite burdens (in footpad ≥ 100-fold)	(Satoskar, et al., 2001)
Ifng **Chr. 10**, 66,75 cM, 118,441 Mb Interferon-gamma	KO mice backcrossed to C57BL/6 for 7 generations	*L. major* (strainWHOM/IR) 10^6 promastigotes inoculated in the hind footpads)	$CD4^+$ T cells develop typical Th2 response ↑ IL-4, IL-5, and IL-13, IgG1, IgE ↓ IgG2a and IgG3 Large lesions development, severe footpad swelling Deaths from 5 to 7 wk post infection occurred Hypertrophy of lymph nodes and spleen Radically increased number of parasites in footpads	(Wang, et al., 1994)
Socs 3 **Chr. 11**, 82,96 cM, 117,966 Mb Suppressor of cytokine signalling 3	129 × C57BL/6 Cre-LoxP system	*L. major* (MHOM/SU/73-5-ASKH) 10^7 promastigotes inoculated subcutaneously in the right footpad	↑ TGF-b1, IL-10, Th3 cells ↓ IL-12 (inhibition of Th1 differentiation) Footpad swelling similar to WT Higher parasite number in lesions remaining 6 wk post infection	(Kinjyo, et al., 2006)
Lgals3 **Chr. 14**, 24,60 cM, 47,367 Mb Lectin, galactose binding, soluble 3	KO mice backcrossed to BALB/c for nine generations	*L. major* LV 39 1×10^7 promastigotes were inoculated into the one hind footpad	↑ IL-10, TGF-β, Notch1, Hes-1 (Notch target gene), Treg cells Increased footpad swelling from 5^{th} wk post infection and number of parasites in lesions from 2^{nd} wk post infection	(Fermino, et al., 2013)

Mapk8 (Jnk1) **Chr. 14**, 20,22 cM, 33,377 Mb J-N (Janus)-terminal kinase	129 × C57BL/6	*L. major* WR309 2×10^6 promastigotes inoculated in to the right hind footpad	↑ IL-5, IL-13, Th2 dependent IgG1 Normal NO and IL-12 production Ability to develop Th1 but simultaneous generation of Th2 response Pattern of lesion formation almost identical to the one observed in BALB/c	(Constant, et al., 2000)
Socs1 **Chr. 16**, 5, 81 cM, 10,783 Mb Suppressor of cytokine signaling-1	C57BL/6 × 129/Sv backcrossed to C57BL/6 mice for ≥10 generations	*L. major* LRC-L137 (MHOM/IL/67/Jericho II) 1×10^4 V121 promastigotes inoculated intradermally into the pinna of the ear	$SOCS1^{+/-}$ led to normal Th1 response (↑ IFNγ, ↓ IL-4, IL-10) but developed significantly large progressive lesions Similar parasite burden to one observed in WT $SOCS1^{-/-}$ ($IFN\gamma^{-/-}$) fatal inflammatory liver disease an overall inability to resolve the disease	(Bullen, et al., 2003)
Bcl6 **Chr. 16**, 15,26 cM, 23,965 Mb B-cell lymphoma 6	129 × C57BL/6	*L. major* strain V1 (MHOM/IL/ 80/Friedlin) 10^6 promastigotes innoculated subcutaneously in the right hind footpad	↑ IL-4, IL-5, IL-13 ↓ IFN-γ Early deaths (5 wk post infection) occurred Severe footpad swelling, skin ulcers and tissue necrosis(similar to BALB/c) Number of parasites in lymph nodes comparable to BALB/c $BCL6^{-/-}$ $IL4^{-/-}$ mice susceptible $BCL6^{-/-}$ $STAT6^{-/-}$ mice resistant	(Dent, et al., 1999)
***H-2b* (*MHC II*)** **Chr. 17**, cytoband B-C Major histoncompatibility complex class II	KO mice backcrossed to C57BL/6 for 5 generations	*L. major* LV 39 10^5 promastigotes inoculated in the hind footpad	↑ activity of CD8 T cells ↓ CD4 T cells, IFN-γ Large lesion development in comparison with WT High number of parasites in lesions remains 65 days post infection while in WT no parasites detected at this time Early infection controlled but later exacerbated	(Chakkalath, et al., 1995)
***Ier3* (IEX-1)** **Chr. 17**, 18.70 cM, 35, 821 Mb Immediate early response 3	129Sv × C57BL/6	*L. major* Friedlin V1 (MHOM/IL/80/FN) 1×10^6 metacyclic promastigotes inoculated intradermally into the ear	↑ IL-17, IL-10 ↓Treg cells, TNF-α, NO No effect on Th polarization Progressive lesion development and delayed healing phenotype Tissue damage began 4 wk post infection (100-fold increase in the parasite load in the infected ears)	(Akilov, et al., 2009)

Tnf **Chr. 17**, 18, 59 cM, 35,199 Mb Tumour necrosis factor	C57BL/6	*L. major* promastigotes (MHOM/IL/81/FEBNI) 3×10^6 promastigotes inoculated subcutaneously into footpad	Delay of the induction of Th1 cells Accumulation of infected cells in their lesions ↓ NO Moderate increase in lesion size, no ulceration observed Parasite burden comparable to WT but 6 wk post infection significant increase detected Deaths from 6 to 9 wk post infection occurred	(Wilhelm, et al., 2001)
Fas/FasL **Chr. 19** (*Fas*), 29,48 cM, 34,290 Mb **Chr. 1** (*Fasl*), 69,95 cM, 161,780 Mb (APO1) Apoptosis antigen 1	C57BL/6 and C3H/HeN (*Fas*-deficient mice on a C57BL/6 background only)	*L. major* LV 39 (MRHO/SU/59/P-strain) 2×10^6 promastigotes inoculated subcutaneously in one hind footpad	No impairment in IFN-γ and IL-4 production ↓ NO Ability to develop Th1 response Progressive lesion development Higher parasite numbers in lesions (reaching values 100-fold higher to WT 3 months post infection) Phenotype was not as severe as in BALB/c mice	(Conceicao-Silva, et al., 1998)
Cd40lg **Chr. X**, 31,21 cM, 57,212 Mb CD40 ligand	C57BL/6 × 129/ J	*L. major* (MHOM/IL/80/Friedlin strain) $2 - 5 \times 10^5$ promastigotes inoculated in the hind footpad	↑ IL-4, IL-10 ↓ IFN-γ, IL-2, IL-12 Failure to develop Th1 response Progressive ulcerating lesions development Increased footpad swelling	(Campbell, et al., 1996)
Gene KO enhancing the resistance	**Mouse strain**	**Parasites**	**Outcome in comparison to WT unless otherwise stated**	**References**
Il17 **(Ctla-8)** Chr. 1, 6,45 cM, 20,730 Mb Cytotoxic T lymphocyte associated antigen 8	KO mice backcrossed to BALB/c for ≥ 10 generations	*L. major* V1(MHOM/IL/80/Friedlin) 1×10^3 or 2×10^5 promastigotes inoculated intradermally in the ear	Th2 development ↓ CXCL2, neutrophils in site of infection Similar IFN-γ, IL-4, IL-10 as in BALB/c Similar DC recruitment as in resistant mice Possible role in neutrophil recruitment Significantly smaller lesions and lower parasite burdens Inability to resolve the lesions contrary to C57BL/6 mice	(Kostka, et al., 2009)
Il1a **Chr. 2**, 62,90 cM, 129,299 Mb Interleukin-1	BALB/c	*L. major* (MHOM/IL/80/Friedlin) 10^7 promastigotes inoculated subcutaneously at the base of the tail	↑ IL-12, IL-18, IFN-γ ↓ IL-4, IL-6 IL-10, IL-17 Less or no parasites detected in both liver and spleen Only late, moderate splenomegaly and mild signs of hepatitis Minimum nodules development No significant change in number of T cells and macrophages	(Voronov, et al., 2010)

***Itgam* (*CR3*), CD11b chain** **Chr. 7**, 69.93 cM, 128,062 Mb Complement Receptor 3 Integrin alpha M	129/J × C57BL/6 backcrossed to both BALB/c and C57BL/6 for 8 times	*L. major* (MHOM/IL/80/Friedlin) 1×10^3 or 105 promastigotes inoculated intradermally in the ear	Similar profiles of Th1 and Th2 cytokine production as well as parasite burdens in BALB/c WT and KO mice From week 8 lesions began to decrease in size Reduced tissue damage and delayed necrosis Infection is less severe in CD11b-deficient mice than in BALB/c WT animals but parasites are probably able utilize other receptors as mechanisms of entry	(Carter, et al., 2009)
Il13 **Chr. 11**, 31,98 cM, 53,631 Mb Interleukin-13	KO mice backcrossed to C57BL/6 for 6 generations BALB/c	*L. major* LV39 2×10^6 promastigotes inoculated in the hind footpad	↑ IL-5 and ↓ IFN-γ (when overexpressed in resistant mice) Overexpression of IL-13 led to inability to resolve the infection independently on IL-4 IL-$13^{-/-}$ and IL-$13^{-/-}$IL-$4^{-/-}$ mice were able to control the infection and by day 56 had normal footpads Additive effect of deleting both IL-4 and IL-13 on resistance	(Matthews, et al., 2000)
Ccl2 (MCP-1) **Chr. 11**, 49,82 cM, 82,035 Mb Monocyte chemoattractant protein-1	BALB/c	*L. major* 2×10^6 promastigotes innoculated subcutaneously in the hind footpad	↓ IL-4, IL-5, IL-10 No impairment of IFN-γ, IL-2, IL-12 production KO mice are nearly Th2 deficient Significantly less footpad swelling than BALB/c Delayed healing phenotype in comparison with C57BL/6 Phenotype between completely susceptible and completely resistant strains	(Gu, et al., 2000)
Ahr **Chr. 12**, 15,78 cM, 35,497 Mb Aryl Hydrocarbon Receptor	C57BL/6	*L. major* LV39 3×10^6 promastigotes inoculated in the hind footpad	Firstly ↓IL-12 and ↑ IL-10 later typical Th1 response ↑ IFN-γ, IL-12, TNF-α ↓ IL-4, IL-10 Decrease in number of parasites in footpads Decrease in lesion size 4 wk post infection	(Elizondo, et al., 2011)

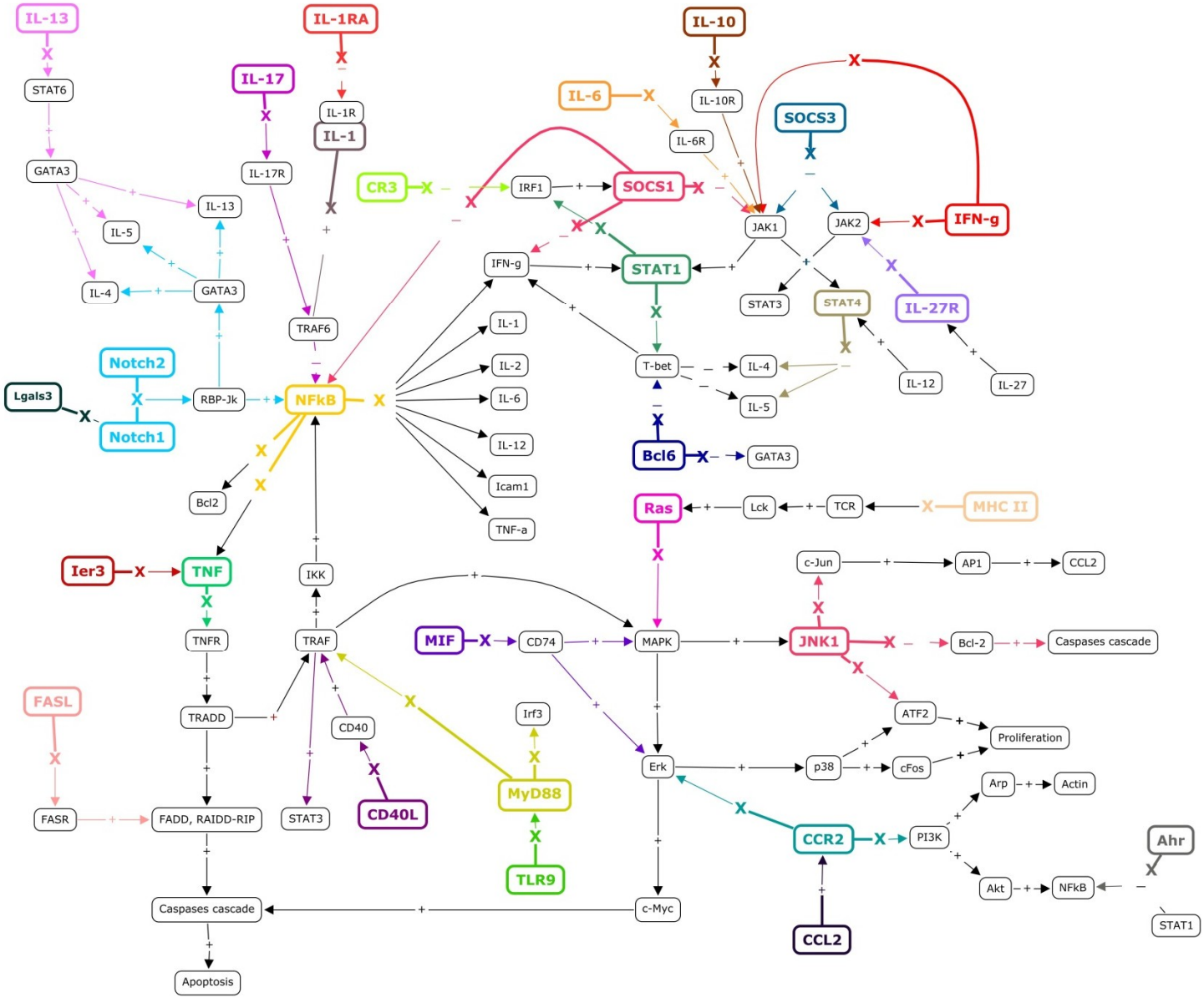
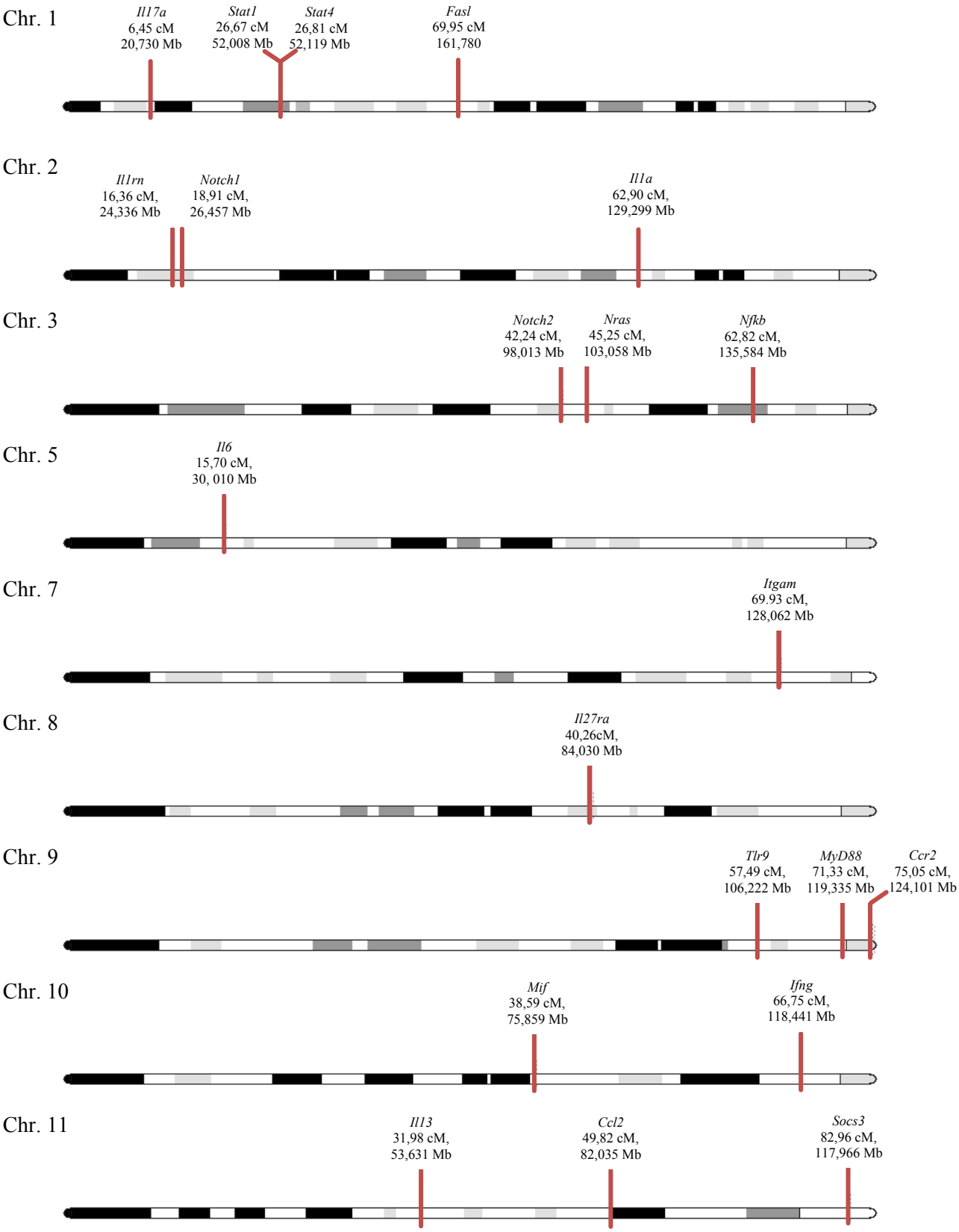


Figure 5: An overview of products of knocked out genes and the pathways in which they are involved. The map was created based on the information took from the articles of particular KO and from Pathwaymaps of Thomson Reuters

Abbreviations used in Figure 5

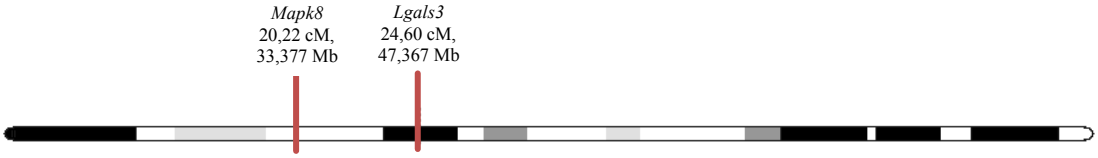
Ahr	Aryl hydrocarbon receptor
Akt	Thymoma viral proto-oncogene
AP1	Activator protein 1
Arp	Arp lymphoid/erythroid hyperplasia
ATF2	Activating transcription factor 2
Bcl2, 6	B cell leukemia/lymphoma 2, 6
CCL2	Chemokine (C-C motif) ligand 2
CCR2	Chemokine (C-C motif) receptor 2
CD40, 74	Cluster of differentiation 40, 74 antigen
CD40L	Cluster of differentiation 40 ligand
cfos	FBJ osteosarcoma oncogene
c-Jun	Jun proto-oncogene
c-Myc	Myelocytomatosis oncogene
CR3 (Itgam)	Integrin alpha M
Erk	Extracellular signal-regulated kinase
FADD	Fas-associated via death domain
FASL	Fas ligand
FASR	Fas receptor
GATA3	GATA binding protein 3
Icam1	Intercellular adhesion molecule 1
Ier3	Immediate early response 3
IFN-γ	Interferon gamma
IκK	IκB kinase
IL-*x*	Interleukin x
IL-*x*R	Interleukin receptor *x*
Irf	Interferon regulatory factor 1
Jak1, 2	Janus kinase 1, 2
Jnk1 (Mapk8)	Mitogen-activated protein kinase 8
Lck	Lymphocyte protein tyrosine kinase
Lgals3	Lectin, galactose binding, soluble 3
Mapk	Mitogen-activated protein kinase
MHC II	Major histoncompatibility complex class II
Mif	Macrophage migration inhibitory factor
MyD88	Myeloid differentiation primary response gene 88
NF-kB	Nuclear factor of kappa light polypeptide gene enhancer in B cells
p38	p38 mitogen-activated protein kinase
PI3K	Phosphoinositide 3-kinase
Raidd (Cradd)	CASP2 and RIPK1 domain containing adaptor with death domain
Ras	Rat sarcoma virus oncogene
RBP-Jk	Recombination signal binding protein for immunoglobulin kappa J
region	
Rip	Regulation of phenobarbitol-inducible P450
Socs	Suppressor of cytokine signaling
Stat	Signal transducer and activator of transcription
T-bet (Tbx21)	T-box 21
TCR	T-cell receptor
TLR	Toll-like receptor
Tnf	Tumor necrosis factor
TnfR	Tumor necrosis factor receptor
Tradd	TNFR-associated via death domain
Traf	TNF receptor-associated factor

Table 2: Approximate positions of knocked-out genes on the chromosomes



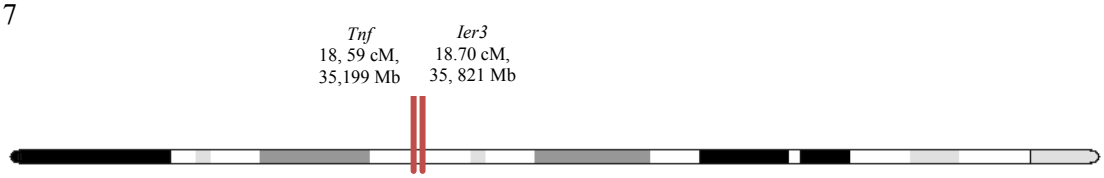
Chr. 12

Chr. 14

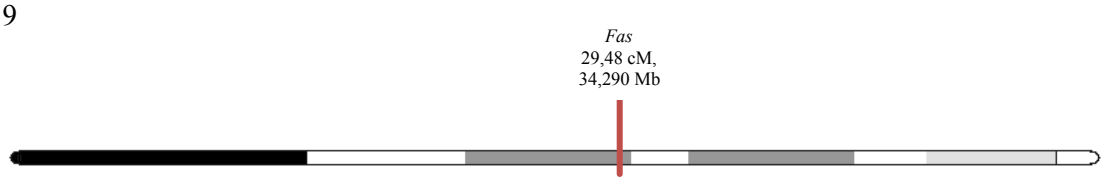


Chr. 16

Chr. 17



Chr. 19



Chr. X